Evaluation of intervention measures to control *Campylobacter jejuni* in broiler chickens

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Thesis submitted in the fulfillment of the requirements for the degree of Doctor in Veterinary Sciences (PhD), Faculty of Veterinary Medicine, Ghent University, 2012

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LIST OF ABBREVIATIONS

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°C	degrees celsius
µ*M*	micromolar
ABC	ammoniumbicarbonate
ACN	acetonitril
ANOVA	one-way analysis of variance
ABC	ATP-binding cassette
BB	blocking buffer
BLAST	basic local alignment search tool
BSA	bovine serum albumin
$CaCl_2$	calcium chloride
C. jejuni	*Campylobacter jejuni*
cCIN	micro-encapsulated *trans*-cinnamaldehyde
cfu	colony-forming units
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EB	extraction buffer
EC	Ethical Committee
EGCG	epigallocatechin gallate
ELISA	enzyme-linked immunosorbent assay
ESI-QUAD-TOF	electrospray ionization quadripole time of flight
EU	European Union
FCA	Freunds' complete adjuvans
FDA	Food and Drug Administration
FIA	Freunds' incomplete adjuvans
FoodNet	Foodborne Diseases Active Surveillance Network
GBS	Guillain-Baré syndrome
GI	gastro-intestinal
GRAS	generally recognized as safe
h	hour
HBSS	Hanks' balanced salt solution
HCl	hydrochloric acid

HEPES	N-2-hydroxyethylpiperazine-N9-2-ethanesulfonic acid
HPLC	high-performance liquid chromatography
HRP	horseradish peroxidase
IgY	immunoglobulin Y
kDa	kilodalton
log	logaritm
LOS	lipooligosaccharide
LPS	lipopolysaccharide
Mbp	megabasepairs
mCCDA	modified charcoal cefoperazone deoxycholate agar
MCFA	medium-chain fatty acids
MCP	methyl-accepting chemotaxis protein
MHB	Mueller-Hinton broth
MIC	minimal inhibitory concentration
min	minute
mL	milliliter
m*M*	millimolar
MOMP	major outer-membrane protein
MS	mass spectrometry
NaCl	sodium chloride
NaOH	sodiumhydroxide
NB2	Nutrient Broth No.2
OD	optical density
P	p-value
PBS	phosphate-buffered saline
PDAC	plant-derived antimicrobial compound
PFGE	pulsed-field gel electrophoresis
pi	post-inoculation
ppm	parts per million
RAPD	random amplification of polymorphic DNA
rpm	rounds per minute
RT	room temperature
SACS	surface-accessible carbohydrate structure
SCFA	short-chain fatty acids

SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
spp	species
TAT	twin-arginine translocase
TBS	tris-buffered saline
UK	United Kingdom
US	United States
V	volt
vol	volume
WB	washing buffer/western blot
wt	weight

CHAPTER 1: LITERATURE REVIEW

Adapted from:

Hermans D, Pasmans F, Messens W, Martel A, Van Immerseel F, Rasschaert G, Heyndrickx M, Van Deun K, Haesebrouck F. (2011). Poultry as a host for the zoonotic pathogen *Campylobacter* jejuni. *Vector Borne Zoonotic Dis*, 12:89-98;

Hermans D, Van Deun K, Martel A, Van Immerseel F, Messens W, Haesebrouck F, Heyndrickx M, Pasmans F. (2011). Colonization factors of *Campylobacter jejuni* in the chicken gut. *Vet Res*, 42:82. doi:10.1186/1297-9716-42-82;

Hermans D, Pasmans F, Heyndrickx M, Van Immerseel F, Martel A, Van Deun K, Haesebrouck F. (2011). A tolerogenic mucosal immune response leads to persistent *Campylobacter jejuni* colonization in the chicken gut. *Crit Rev Microbiol*, 38:17-29 &

Hermans D, Van Deun K, Messens W, Martel A, Van Immerseel F, Haesebrouck F, Rasschaert G, Heyndrickx M, Pasmans F. (2011). *Campylobacter* control in poultry by current intervention measures ineffective: urgent need for intensified fundamental research. *Vet Microbiol*, 152:219-228.

CHAPTER 1: LITERATURE REVIEW

1.1 POULTRY AS A HOST FOR ZOONOTIC *CAMPYLOBACTER* SPP.

1.1.1 General introduction

Campylobacter species are Gram-negative bacteria that belong to the family of the *Campylobacteraceae*. These micro-organisms generally possess a single polar flagellum at one or both sides of their cell end, promoting motility. Campylobacters are unable to ferment or oxidize carbohydrates and therefore depend on amino acids and Krebs cycle intermediates as primary energy sources. The bacteria are microaerophilic, only growing at reduced oxygen levels, and have a minimal growth temperature of over 30°C. Both these growth requirements make it unlikely for campylobacters to multiply in the outside environment (Snelling *et al*. 2005). Instead, they seem to be highly adapted to some warm-blooded animal reservoirs, in which they primarily occur as commensals.

In humans, campylobacters are now the leading cause of bacterial gastro-enteritis in many developed countries. Campylobacters generally do not cause large outbreaks of gastro-intestinal illness since over 90% of campylobacteriosis cases in humans are sporadic (Snelling *et al*., 2005). Most *Campylobacter* infections are self-limiting and thus do not require antimicrobial therapy (Butzler, 2004). These infections generally last for up to seven days and are accompanied with extremely diverse clinical manifestations, ranging from a complete absence of symptoms to fulminating sepsis. In a small number of cases, however, the infection may develop into long-term complications and even death. These post-infectious complications include arthritis, Reiter syndrome and Guillain-Barré syndrome (GBS), an acute auto-immune disease affecting the peripheral nervous system leading to muscle weakness and sometimes paralysis, and that can even be life-threatening (Butzler, 2004; Snelling *et al*. 2005). Although during recent years the number of registered campylobacteriosis cases has declined slightly in some parts of the world outside the EU (Ailes *et al*., 2008), the overall disease burden is still noteworthy and registered cases in the EU are steadily increasing (EFSA, 2012). The true incidence of campylobacteriosis in industrialized countries is uncertain since many unreported infections occur for every diagnosed case (Havelaar *et al*., 2012). Within the European Union, campylobacteriosis has been the most frequently reported zoonotic disease in humans as from 2005. In 2010, the overall EU notification rate was 48.6 cases per 100,000 inhabitants (EFSA, 2012). Thanks to

a significant decrease in campylobacteriosis cases compared to previous years (2005 - 2009), Belgium remained below this average with a notification rate of 28.0 per 100,000 inhabitants in 2010 (EFSA, 20011a; 2012). In the United States, the Foodborne Diseases Active Surveillance Network (FoodNet) reported an incidence of culture-confirmed *Campylobacter* infections in the FoodNet sites of 12.7 per 100,000 persons in 2006 (Ailes *et al*., 2008). These numbers represent a 30% decline compared to the 1996 situation, but the incidence still remains above the national health objective. Most other regions of the world report a higher disease incidence, with strikingly high numbers in New Zealand in 2003 of almost 400 cases per 100,000 people (Baker *et al*., 2007). One group of campylobacters, the "thermotolerant" or "thermophilic" *Campylobacter* species (containing *C. jejuni*, *C. coli*, *C. lari*, *C. upsaliensis* and *C. helveticus* and for simplicity hereafter referred to as *Campylobacter*) are especially associated with human gastro-intestinal disease (Snelling *et al*., 2005). *Campylobacter* enteritis in humans is, however, mainly caused by *C. jejuni* (EFSA, 2012). In 2010, 35.7% of the isolates were identified as *C. jejuni*, representing 93.4% of the cases characterized at species level. *C. coli*, *C. lari* and *C. upsaliensis* accounted only for respectively 2.3%, 0.22% and 0.006% of the isolates. The remainder of the isolates included other species or were not speciated or not known. *C. jejuni* is able to infect humans at doses as low as 500 colony-forming units (Snelling *et al*. 2005).

C. jejuni contains two subspecies, namely ssp. *doylei* and ssp. *jejuni*. The pathogenic role of *C. jejuni* ssp. *doyley* is unknown. Therefore, from here on, *C. jejuni* refers to *C. jejuni* ssp. *jejuni*. *C. jejuni* are S-shaped rods (0.2-0.8 µm wide and 0.5-5.0 µm long) with a small genome (1.6-1.7 Mbp). This feature might possibly explain their stringent growth requirements. Besides being asaccharolytic, *C. jejuni* does not have lipase or lecithinase activity. In addition, these bacteria do not grow below pH 4.9 and require complex growth media to allow culturing (Snelling *et al*. 2005).

1.1.2 *Campylobacter* prevalence in animals and the environment

Paradoxally, despite their fastidious, fragile nature, campylobacters are highly prevalent in the environment and can survive for prolonged periods both inside and outside a suitable host (Newell, 2002; Murphy *et al*., 2006). How the microorganism copes with stresses encountered in the environment still remains enigmatic but clearly, campylobacters developed some survival mechanisms to overcome these stressors (Murphy *et al*., 2006). The presence of highly mutable sites in the *C. jejuni* genome (see 1.2.1) is responsible for its rapid adaptation

in a novel host (Jerome *et al*., 2011). Campylobacters can be frequently found in surface water and is part of the natural intestinal microbiota of a wide range of wild and domestic animals, especially poultry (Newell, 2002, Whyte *et al*., 2004; Abulreesh *et al*., 2006; Young *et al*., 2007; Ogden *et al*., 2009, Jokinen *et al*., 2011). The estimated *Campylobacter* prevalence in poultry and non-poultry farm animals depends on season, age of animal, flock or herd size and type, diet, husbandry practices and geography, with overall *C. jejuni* being the most isolated species (Kuana *et al*., 2008; McDowell *et al*., 2008; Zweifel *et al*., 2008; Ellis-Iversen *et al*., 2009; Messens *et al*., 2009; Näther *et al*., 2009; EFSA, 2010b; EFSA, 2012; Jorgenson *et al*., 2011).

1.1.3 Poultry as a natural host for zoonotic *Campylobacter*

Chickens are a natural host for *Campylobacter* species (EFSA, 2012). The probability of a flock to become colonized increases during rearing, resulting in on average 60% to 80% of the analyzed broiler flocks to be positive for *Campylobacter* species in general at slaughter age worldwide (Herman *et al*., 2003; Rasschaert *et al*., 2006; Kuana *et al*., 2008; Reich *et al*., 2008; EFSA, 2010b). Striking differences in EU prevalence do exist. In 2008, almost all broiler batches in some Northern-European countries like Estonia, Norway and Finland were free of *Campylobacter*, while a strikingly high prevalence of 100% was reported for Luxembourg. With a prevalence of 31.0% *Campylobacter*-colonized broiler batches in 2008, Belgium stayed below the average EU prevalence of 71.2%. In general, *C. jejuni* was found to be the dominating species in broilers, although in some Southern European Member States, especially Malta and Luxembourg, *C. coli* was the most commonly isolated *Campylobacter* species (EFSA, 2010c). Colonized poultry flocks might contaminate the surrounding environment by which *Campylobacter* is able to spread further and contaminate other farms or humans (Jonsson *et al*., 2010). Since *C. jejuni* is the most important species causing disease in humans, the focus of this thesis will be primarily on this species.

1.1.4 Colonization of broiler chickens with *C. jejuni* at the farm

From day-of-hatch until the broiler chickens are transported to the abattoir, the animals can encounter several risk factors contributing to their colonization with *C. jejuni*. As a consequence, the *Campylobacter* epidemiology in broiler flocks is quite complex. An overview of the risk factors involved in the environmental transmission of *Campylobacter* to broiler flocks is given in **table 1**.

1.1.4.1 *Initial broiler flock colonization*

Initial colonization of broiler chickens probably occurs through horizontal transmission from the environment, while vertical transmission from breeder hens or carryover of infection from a positive flock to a new flock in the same house, after cleansing and disinfection, are considered to be unlikely (van de Giessen *et al*., 1992; Jacobs-Reitsma *et al*., 1995; Bull *et al*., 2006; Patriarchi *et al*., 2011). Indeed, carryover of *C. jejuni* subtypes between broiler flocks in the same house seems to occur only rarely (Barrios *et al*., 2006; Colles *et al*., 2008; McDowell *et al*., 2008). Persistent clones in the outside environment can, however, be responsible for repeated infection of multiple broiler flock rotations (Petersen & Wedderkopp, 2001; Wedderkopp *et al*., 2003; Kudirkiene *et al*., 2010).

Colonization of broiler chickens with *C. jejuni* is influenced by many factors including source of the microorganism, the infecting dose and age of the animal (Stas *et al*., 1999). Most broiler flocks become colonized only at an age of two to four weeks, after which the infection rapidly spreads to all birds, which remain colonized until slaughter (Jacobs-Reitsma *et al*., 1995; Stern *et al*., 2001b; Stern, 2008; van Gerwe *et al*., 2009). The likelihood of a flock to become colonized with *Campylobacter* increases with higher slaughter age (Berndtson *et al*., 1996b; Barrios *et al*., 2006). Also the time of year clearly has an influence. In Germany and the UK, the risk for broilers to become colonized with *Campylobacter* is highest during the summer months (McDowell *et al*., 2008; Ellis-Iversen *et al*., 2009; EFSA, 2010d; Ellerbroek *et al*., 2010; Jorgenson *et al*., 2011) and a coincident seasonality of infections in chickens and humans has been shown (Meldrum *et al*., 2005). A significant relationship was observed between several climatic factors (such as environmental temperature and amount of sunshine and rainfall) and *Campylobacter* prevalence in UK broiler flocks prior to first partial or full depopulation (Jorgenson *et al*., 2011), which could explain this observed seasonality although other factors, such as husbandry practices and biosecurity, may also be important (Allen *et al*., 2011). Finally, an increased flock size was associated with a higher probability of that flock to be colonized with *Campylobacter* (Berndtson *et al*., 1996b; Barrios *et al*., 2006).

1.1.4.2 *Sources for horizontal* Campylobacter *transmission to broiler flocks*

Colonized livestock and free-living animals are an important risk factor for transmitting *C. jejuni* to broiler flocks as *C. jejuni* genotypes from cattle and laying hens, present at poultry farms, can also be found in the broiler flocks (van de Giessen *et al*., 1996; Ridley *et al*., 2008a; Zweifel *et al*., 2008; Ellis-Iversen *et al*., 2009; Allen *et al*., 2011), while pigs are a source for *C. coli* (Zweifel *et al*., 2008). Bovine *Campylobacter* isolates are indeed able to

efficiently colonize chickens (Hanel *et al*., 2009) and might thus be a source for broiler chicken infection, although they differ significantly from chicken and human isolates by a reduced prevalence of two genetic markers especially, the *dmsA* and γ-glutamate transpeptidase (*ggt*) gene (see 1.2.2.10), important for persistent colonization of *C. jejuni* in chickens (Barnes *et al*., 2007; Gonzalez *et al*., 2009). Also several other studies revealed the presence of identical *C. jejuni* clones in bovines, chickens and humans (Nielsen *et al.*, 1997; On *et al.*, 1998; Gilpin *et al.*, 2008; Ragimbeau *et al.*, 2008; Hakkinen *et al.*, 2009; Huang *et al.*, 2009). Recently, it was shown by molecular typing that *Campylobacter* strains from a broiler house and from an adjacent dairy farm were similar to those subsequently detected in the flock, indicating the importance of horizontal transmission and the risk of transmission of *Campylobacter* on multispecies farms (Ridley *et al*., 2011). This study also indicated that bovine fecal *Campylobacter* strains can colonize chickens, which was confirmed later on by Patriarchi *et al*. (2011). On a German farm, indistinguishable isolates were found in different flocks during the same rearing period (Ellerbroek *et al*., 2010). This suggests that *Campylobacter* strains might be transmitted from one broiler flock to another or might point toward a common external source infecting multiple broiler flocks at the same farm.

Also rodents, flies and their larvae are potential vectors for *C. jejuni* transmission to broiler flocks (Berndtson *et al*., 1996a; Hald *et al.*, 2004; Nichols, 2005; Hald *et al.*, 2008; Hazeleger *et al.*, 2008). An association has been shown between the presence of rodents on farms and an increased risk for flocks to become colonized with *Campylobacter* (McDowell *et al*., 2008).

Another important source of infection is contaminated surface water as genotypes found in broilers can sometimes be detected in water puddles and ditch water as well, before the flocks are colonized (Bull *et al*., 2006; Messens *et al*., 2009). *C. jejuni* survival in water is promoted by several factors, including biofilm formation and possibly the viable but non-culturable state, in which *C. jejuni* enters when outside a suitable host (Sparks, 2009).

Personnel and farm equipment such as trucks, forklifts, pallets, crates and footwear have also been identified as potential sources of *C. jejuni* infection of broilers (Ramabu *et al.*, 2004). Also, broiler flocks on farms with three or more broiler houses, low frequency of footbath disinfectant change and decreased cleanliness of the broiler house ante-room, have an increased risk to become colonized (McDowell *et al*., 2008).

Finally, partial depopulation/thinning of broiler flocks (early removal of a part of the birds) has been implicated as a potential risk factor for *Campylobacter* colonization of the remainder of the animals of these flocks due to difficulties in maintaining biosecurity during thinning (Patriarchi *et al*., 2011, EFSA, 2011b). Allen *et al*. (2008) showed that during the thinning

procedure particular *C. jejuni* strains can spread from one farm to another (nearby farm) when sharing the same bird-catching personnel and/or vehicles. However, in several other studies, no increased risk associated with this process has been shown (Russa *et al*., 2005; Barrios *et al*., 2006; Näther *et al*., 2009).

1.1.4.3 *Transmission through the flock*

Once flock colonization occurs, bird-to-bird transmission within flocks is very rapid. A recent mathematical model revealed a transmission rate of 2.37 (van Gerwe *et al*., 2009) new cases per colonized chick per day. This implies that in a flock of 20,000 broilers, the prevalence of *Campylobacter* would increase from one infected bird to 95% within the week after the first bird is infected. Indeed, in a study by Stern *et al*. (2001), the majority (95% to 100%) of birds in a flock was colonized within seven days after contact with a single (*Campylobacter*-colonized) seeder bird, regardless of the age of the animals. Drinking water and feed are believed to play an important role in this fecal-oral spread through the flock (Evans, 1992; Gregory *et al.*, 1997; Herman *et al.*, 2003; Newell & Fearnley, 2003; Sparks, 2009). Once a flock is colonized, the nipple water is often contaminated with *C. jejuni* strains that are indistinguishable from those isolated from the broilers, indicating the importance of drinking water in transmitting this zoonotic pathogen throughout the flock (Messens *et al.*, 2009).

1.1.4.4 *The effect of transportation*

Transport-induced stress has been shown to increase both the *Campylobacter* load (by 0.7 $\log_{10}$ cfu/g) in broiler ceca (Stern *et al.*, 1995), as well as its excretion rates in broiler feces (by 0.8 $\log_{10}$ cfu/g) after transport (Whyte *et al.*, 2001). Transport crates are often still contaminated with *Campylobacter* when reused because crate decontamination processes are mostly ineffective. Contaminated crates can lead to external contamination of birds during transport of a negative flock to the processing plant (Slader *et al.*, 2002; Herman *et al.*, 2003; Hansson *et al.*, 2005; Rasschaert *et al.*, 2007). However, there is still controversy about the possible role of transport crates in transmission of *Campylobacter*. Evidence for intestinal colonization or co-colonization due to transport in *Campylobacter*-contaminated containers was not found by Rasschaert *et al.* (2007). On the other hand, *C. jejuni* genotypes commonly associated with chickens were dominantly found on transport equipment and persisted through the decontamination process (consisting of washing with water and peracetic acid sanitization), indicating that improperly disinfected transport crates are involved in *Campylobacter* contamination of poultry flocks (Hastings *et al.*, 2010; Patriarchi *et al*., 2011).

1.1.4.5 *Combined hygienic approach to prevent flock colonization*

It is clear that lots of risk factors are involved in the environmental transmission of *Campylobacter* to broiler flocks (**table 1**). Possibly these factors are intimately linked with each other. The increased temperature during summer months for instance could promote the presence of flies and rodents at the farm, while increased rain fall can create water puddle reservoirs in which *C. jejuni* can persist and transmit to other vectors, such as cattle and other farm animals, birds, flies and rodents (Jorgenson *et al*., 2011). Therefore, source attribution for *Campylobacter* colonization in poultry flocks is not straightforward and only a combined approach of properly implemented hygienic measures in all of these areas will be capable to significantly reduce the number of *Campylobacter*-colonized flocks. This combined approach must aim at minimizing the probability that *Campylobacter* enters the broiler room (by rodent and insect control, footbath disinfection for personnel working in the broiler room and drinking water treatment; see 1.3 for on-farm *Campylobacter* control measures) and preventing cross-contamination during transport (by decontamination of transport crates) and slaughter (see below).

1.1.5 Broiler carcass contamination with *Campylobacter*

A significant correlation exists between the *Campylobacter* numbers in ceca of broiler chickens during rearing and bacterial counts on their carcasses after processing (Herman *et al.*, 2003; Rasschaert *et al*., 2006; Rosenquist *et al*., 2006; Reich *et al*., 2008). Worldwide, an average prevalence of *Campylobacter*-contaminated poultry carcasses is reported to be in the range of 60% to 80% (Suzuki & Yamamoto, 2009; EFSA, 2010c; Mullner *et al.*, 2010). Similarly as in live animals of a poultry flock, also on poultry carcasses *C. jejuni* is the predominating species (Rasschaert *et al.*, 2006; Kuana *et al.*, 2008; Suzuki & Yamamoto, 2009; EFSA, 2010b; EFSA, 2010c, EFSA, 2012). Carcass contamination occurs during defeathering and evisceration, by contaminated feces leaking from the cloaca and visceral rupture of ceca carrying a high *Campylobacter* load (Berrang *et al.*, 2001; Allen *et al.*, 2008; Boysen & Rosenquist, 2009).

The source of the majority of *Campylobacter* genotypes contaminating carcasses during the slaughter process is probably the live flock (Herman *et al.*, 2003; Rosenquist *et al.*, 2003; Normand *et al.*, 2008; Colles *et al.*, 2010; EFSA, 2010d), but carcasses can also become contaminated by cross-contamination between birds within a flock and between flocks slaughtered successively (Herman *et al.*, 2003; Rosenquist *et al.*, 2003; Rasschaert *et al.*,

2006) and even between carcasses of different flocks slaughtered in the same area (Normand *et al*., 2008), increasing the diversity of *Campylobacter* genotypes isolated from a flock after slaughter (Colles *et al.*, 2010). *Campylobacter*-colonized flocks contaminate the abattoir environment upon entering the room and as a consequence *Campylobacter* can be isolated at all stages of the processing line (Ellerbroek *et al*., 2010). *C. jejuni* is able to survive overnight on these processing equipment surfaces, even after cleaning (using high-pressure water and alkaline-chlorinated molecules or a neutral detergent) and disinfection (using quaternary ammonium compounds combined with gluteraldehyde or with poly (hexamethylene biguanide) chlorohydrate) (Peyrat *et al.*, 2008). Therefore, surviving strains may be a source of poultry carcass contamination of subsequent (*Campylobacter*-free) flocks (Newell *et al.*, 2001; Miwa *et al.*, 2003). Due to their stringent growth requirements, campylobacters are unable to grow in/on these carcasses and meat preparations.

1.1.6 Transmission of *Campylobacter* from broilers to humans

Transmission to humans most commonly occurs through consumption and handling of all kinds of foods of animal origin of which the carcasses are contaminated with *Campylobacter* (Berrang *et al.*, 2001; EFSA, 2010c). In industrialized countries handling, preparation and consumption of contaminated chicken meat is considered to be the main source of infection in humans (Berndtson *et al.*, 1992; Friedman *et al.*, 2004). By using genetic typing methods (see 1.1.7) it was evidenced that chicken meat *Campylobacter* isolates can frequently be linked to human cases of campylobacteriosis. However, the overall genotypic diversity between isolates indicates that there are other sources contributing to disease in humans as well (de Haan *et al*., 2010; Thakur *et al*., 2010). Indeed, besides poultry, also non-poultry farm animals can contribute to campylobacteriosis in humans. *Campylobacter* colonization of such animals not only poses a risk of contamination of their carcasses at slaughter, but it can also lead to the contamination of milk and surface water at the farm, as well as colonization of broiler flocks present at these farms (Doyle & Roman, 1982; Stanley & Jones, 2003; Garrett *et al*., 2006; Hannon *et al*., 2009), which are all risk factors for transmitting *C. jejuni* to humans. Also direct contact with cattle, but also pets, in particular puppies with diarrhea, is a possible route of contamination (Tenkate & Stafford, 2001; de Haan *et al*., 2010). Cattle and their direct environment are thus important reservoirs for disease in humans (Kwan *et al*., 2008). Finally, also drinking water as well as raw vegetables, which can become contaminated by cross-contamination by other contaminated food products during preparation but also directly at the

farm, have been implicated as possible sources for human illness (Evans *et al*., 2003; Gardner *et al*., 2011; Verhoeff-Bakkenes *et al*., 2011).

1.1.7 Contribution of the chicken reservoir to campylobacteriosis in humans

Campylobacters generally do not cause large outbreaks of gastro-intestinal illness since over 90% of campylobacteriosis cases in humans are sporadic (Snelling *et al*., 2005).

Due to the wide-spread occurrence of *Campylobacter* spp., its environmental cycle is not very well understood. Moreover, due to the possibility of cross-contamination, tracing the genuine source of *Campylobacter* infections is not straightforward. By using a genetic approach, however, Wilson *et al*. (2008) estimated that 97% of the number of sporadic human campylobacteriosis cases in England is attributable to animals farmed for meat, with chicken and cattle as main sources for *C. jejuni*, indicating that contaminated food products are the principle source for disease in humans. Stern & Kazmi (1989) stated already over two decades ago that a large number of *C. jejuni* serotypes from poultry can frequently be linked to human cases of campylobacteriosis. This was confirmed by later reports using also genetic typing techniques (Nielsen *et al.*, 1997; Nadeau *et al.*, 2002; Zorman *et al.,* 2006; Colles *et al*., 2008; Gonzalez *et al*., 2009; Lindmark *et al*., 2009; Wassenaar *et al*., 2009; Mullner *et al*., 2010), together indicating that certain *C. jejuni* strains circulate between poultry and humans. The observation that during the Belgian dioxin crisis in 1999, a withdrawal of chicken meat from the market in June coincided with a 40% decrease in *Campylobacter* infections during that month (Vellinga & Van Loock, 2002), gave further strength to the hypothesis that poultry is a very important source for transmitting this pathogen to humans. Very recently, evidence of transmission by direct contact with poultry carcasses was given by Friis *et al*. (2010) who isolated identical strains from a poultry abattoir and a person that had developed campylobacteriosis upon entering that abattoir. Thus, there is increasing evidence that the broiler chick is a major reservoir for *C. jejuni* pathogenic to human and that broiler chicken meat contaminated with this zoonotic pathogen is the most important source of disease in humans. For the European situation it was estimated that *Campylobacter*-contaminated chicken meat would be responsible for up to 40% of human campylobacteriosis cases. The chicken reservoir as a whole might even be responsible for up to 80% of the cases, because strains from the chicken reservoir may reach humans by pathways other than food (EFSA, 2010c). As a consequence, eradicating *Campylobacter* from poultry lines could tremendously reduce the number of *Campylobacter*-mediated enteritis cases in humans. Unfortunately, no

effective, reliable intervention measure is available to date to reduce *Campylobacter* colonization in poultry (Hermans *et al*., 2011). In the EU, neither the overall prevalence of this pathogen in chicken retail products, nor the number of reported poultry meat consumption-related human campylobacteriosis cases have been reduced in recent years (EFSA, 2010c; 2012).

Table 1 Risk factors for *Campylobacter* colonization of broiler flocks at the farm

Rearing phase	*Influencing factor*	*Risk factor*	*Reference*
Initial colonization	Source of the microorganism and infective dose	Persistent clones in the environment, high colonizer strains and high infective dose	Stas *et al*., 1999 Petersen & Wedderkopp, 2001 Wedderkopp *et al*., 2003 Kudirkiene *et al*., 2010
	Age of the animals	From 2 weeks onward	Jacobs-Reitsma *et al*., 1995 Herman *et al*., 2003 van Gerwe *et al*., 2009
		Higher slaughter age	Barrios *et al*., 2006
	Flock size	Increased flock size	Berndtson *et al*., 1996b Barrios *et al*., 2006
	Seasonality	Summer months	McDowell *et al*., 2008 Ellis-Iversen *et al*., 2009 Ellerbroek *et al*., 2010
	Applied husbandry practices	Ineffective hygiene measures	McDowell *et al*., 2008 Allen *et al*., 2011
	Sources for horizontal transmission	Other colonized animals on the farm	van de Giessen *et al*., 1996 Ridley *et al*., 2008a Zweifel *et al*., 2008 Ellis-Iversen *et al*., 2009 Hanel *et al*., 2009 Allen *et al*., 2011 Patriarchi *et al*., 2011 Ridley *et al*., 2011
		Rodent and insect carriers	Hald *et al.*, 2004 Nichols, 2005 Hald *et al.*, 2008 Hazeleger *et al.*, 2008 McDowell *et al*., 2008
		Contaminated surface water	Messens *et al*., 2009
		Personnel and farm equipment	Ramabu *et al.*, 2004 McDowell *et al*., 2008

		Partial depopulation?	Allen *et al*., 2008 Patriarchi *et al*., 2011 vs. Russa *et al.*, 2005 Barrios *et al*., 2006 Näther *et al*., 2009 EFSA, 2011b
Transmission through the flock	Bird-to-bird transmission by fecal-oral route	Drinking water and feed	Evans, 1992 Gregory *et al.*, 1997 Herman *et al.*, 2003 Messens *et al.*, 2009 Sparks, 2009
Colonization pattern	Increased intestinal bacterial load	Absence of anti-*Campylobacter* substances	Connerton *et al.*, 2004 El-Shibiny *et al.*, 2005
		Chicken diet	Udayamputhoor *et al.*, 2003
Transportation	Increased intestinal bacterial load and fecal *Campylobacter* excretion rates	Transport-induced stress	Stern *et al.*, 1995 Whyte *et al.*, 2001
		Contaminated transport crates	Slader *et al.*, 2002 Herman *et al.*, 2003 Hansson *et al.*, 2005 Rasschaert *et al.*, 2007 Hastings *et al.*, 2010 Patriarchi *et al*., 2011

1.2 *CAMPYLOBACTER* COMMENSALISM IN CHICKENS: COLONIZATION MECHANISM AND IMMUNE RESPONSE

Broiler chickens colonized with *C. jejuni* carry a large number of bacteria especially in their ceca (generally around 10^6 to 10^8 cfu/g), the predominant site of colonization (Beery *et al*., 1988; Meade *et al*., 2009). Ingestion of *C. jejuni* numbers as few as 35 cfu can be sufficient for successful colonization of chicks (Stern *et al*., 1988). Upon ingestion, the bacterium reaches the cecum and multiplies, resulting in a stable colonizing *Campylobacter* population within 24 hours after entrance (Coward *et al*., 2008; Smith *et al*., 2008). Despite carrying such high *C. jejuni* numbers in their ceca, colonization of chickens with *C. jejuni* does generally neither cause clinical illness nor changes in cecal mucosa morphology (Van Deun *et al*., 2008a; Meade *et al*., 2009b). Despite some reports of *Campylobacter*-induced diarrhea, systemic invasion, growth retardation, jejunal villus atrophy and development of a GBS-like paralytic neuropathy after feeding chicks with *C. jejuni* isolated from a GBS patient (Ruiz-Palacios *et al*., 1982; Sanyal *et al*., 1984; Sang *et al*., 1989; Lam *et al*., 1992; Li *et al*., 1996; Lamb-Rosteski *et al*., 2008), it is generally accepted that *C. jejuni* colonizes the avian gut as a commensal.

1.2.1 Colonization potential of *C. jejuni* isolates in poultry

1.2.1.1 *Colonization phenotypes*

C. jejuni isolates can have different colonization potential (Stern *et al*., 1988; Ringoir & Korolik, 2003; Hanel *et al*., 2009). Isolates from humans have been reported to be less successful in colonizing chickens compared to poultry isolates (Korolik *et al*., 1998; Ringoir & Korolik, 2003). *C. jejuni* isolates from poultry have been divided into three colonization phenotypes. Strains of the first phenotype fail to colonize 14-day-old chickens. In the second phenotype, strains can colonize but are readily eliminated and are classified as transient. The third phenotype contains strains that show efficient and sustained colonization (Korolik *et al*., 1998; Hanel *et al*., 2009). These three colonization phenotypes were found to be stable and independent of *in vivo* passages and the amount of viable bacteria in the inoculum. Although *C. jejuni* strains did show enhanced colonization capacity/potential (i.e. the minimal infective dose required for colonization decreased) after passage through the avian gastro-intestinal (GI) tract, their colonization phenotype did not change (Ringoir & Korolik, 2003). Enhanced colonization capacity and increased virulence after *in vivo* passage through chicks has been

shown in several other studies as well (Sang *et al*., 1989; Cawthraw *et al*., 1996). This variability in colonization capacity, but the fixedness of the colonization phenotype of a given strain indicates that *C. jejuni* genes involved in initial and sustained colonization are not identical. However, in contrast to this stable colonization phenotype, it has been previously reported that after several *in vivo* passages a poorly colonizing isolate was able to consistently colonize chicks (Stern *et al*., 1988).

1.2.1.2 *Genetic variation*

Chicken intestinal colonization may favour genomic changes in *C. jejuni* resulting in different *flaA* types, ribopatterns and pulsotypes (Hanninen *et al*., 1999; Van Deun *et al*., 2007; Hanel *et al*., 2009). Interstrain genetic exchange and intragenomic alterations were shown to occur *in vivo*, even in the absence of selective pressure (de Boer *et al*., 2002). It has been demonstrated that bacteriophage genes are known to be present in the genome of *C. jejuni* and that phages can alter pulsed field gel electrophoresis (PFGE) patterns of this bacterium (Barton *et al*., 2007; Clark & Ng, 2008). Both phage-dependent and -independent rearrangements of the genome result in an enormous antigenic variation among *C. jejuni* isolates (Scott *et al*., 2007a, 2007b). Besides protection against phage predation, this generation of antigenic diversity may play an important role in chicken gut colonization. However, *C. jejuni* strains that underwent rearrangements leading to resistance against virulent bacteriophages were demonstrated to be inefficient colonizers of the chick intestine (Scott *et al*., 2007b). There is still some controversy regarding the genomic instability of *C. jejuni* since Nielsen *et al*. (2001) concluded that many strains were genetically stable as tested by riboprinting, PFGE, random amplification of polymorphic DNA (RAPD) and Penner heat-stable serotyping after *in vitro* and *in vivo* (through mice) passage. Moreover, Manning *et al*. (2001) concluded that this stability could be maintained despite exposure to various environmental conditions over long time periods and covering large distances. Also, it has been suggested that subtype pattern variations in *C. jejuni* leading to phenotypic changes, occur only occasionally during *in vivo* passage (Konkel *et al*., 2007). On the other hand, Ridley *et al*. (2008) observed that, although stable during single cecal colonization of one individual strain, the *C. jejuni* genome can undergo changes upon competitive stress in the avian gut, i.e. when colonized with more than one sero- or genotype at the same time, which is referred to as co-colonization. This leads to pulsotype variants with different colonization capacities. Genetic and phenotypic diversity might play a role in the improved fitness of certain *C. jejuni* strains to survive and colonize another host.

1.2.1.3 *Co-colonization*

Co-colonization is frequently detected in *Campylobacter*-positive flocks, which may be explained by recurring environmental exposure to the bacterium but also by genetic changes within the *C. jejuni* population (van de Giessen *et al*., 1992; Jacobs-Reitsma *et al*., 1995). The dominating strains are replaced throughout the colonization period, probably due to strain-specific immune responses, and it seems that this colonization pattern is mainly determined by the chicken host and not by the host microbiota (Skanseng *et al*., 2007; Ridley *et al*., 2008b). Indeed, different breeds of chicken may differ in their susceptibility to colonization with *C. jejuni* (Stern *et al*., 1990a; Boyd *et al*., 2005). It has been suggested that a paternal effect might be an important genetic factor influencing resistance to *C. jejuni* colonization in broilers (Li *et al*., 2008a). However, there are also other lines of evidence suggesting that external factors are responsible for the *Campylobacter* colonization pattern in broilers. It has been found in artificially inoculated birds that different *C. jejuni* genotypes may compete for colonization leading to a *C. jejuni* succession in broilers (Konkel *et al*., 2007).

1.2.2 Colonization mechanisms of *C. jejuni* in the chicken gut

As in the environment, also in the chicken intestine *C. jejuni* is likely to encounter environmental stressors compromising optimal growth (Murphy *et al*., 2006). The persistent colonization of the chicken GI tract by *C. jejuni* indicates that the bacterium harbours regulatory systems that confer protection toward a hostile environment inside the host. The mechanism by which the bacterium adapts to this "hostile" environment, resulting in successful and persistent colonization, is poorly understood. It is clear, however, that successful colonization of the chicken GI tract is a multifactorial process (Newell, 2002), in which genes involved in all areas of the colonization process of *C. jejuni* play a role. In the next sections, a thorough, up-to-date overview is given on identified colonization factors of *C. jejuni* in the chicken gut, summarized in **table 2**.

1.2.2.1 *Multidrug and bile resistance*

The *Campylobacter* multidrug efflux pump (CME) plays an important role in multidrug resistance in *C. jejuni*, mediating resistance to heavy metals and a broad range of antibiotics and other antimicrobial agents (Lin *et al*., 2002). It is also responsible for resistance to bile salts in the chicken intestinal tract and is therefore essential for successful intestinal colonization in chickens (Lin *et al*., 2003). CME is encoded by the operon *cmeABC* and

consists of a periplasmic protein (CmeA), an inner-membrane efflux transporter (CmeB) and an outer-membrane protein (CmeC). Expression of *cmeABC* in *C. jejuni* is modulated by CmeR, functioning as a transcriptional repressor (Lin *et al*., 2002). In a *cmeR* mutant, one gene in particular was upregulated most compared to the wild type strain: *cj0561c*, encoding a putative periplasmic protein (Guo *et al*., 2008). It is suggested that CmeR directly inhibits the transcription of this gene. The expression of both *cmeABC* and *cj0561c* is strongly induced by bile compounds present in the chicken intestinal tract and expression of Cj0561c is increased over four-fold during chicken colonization (Woodall *et al*., 2005; Guo *et al*., 2008). Inactivation of *cj0561c* and loss-of-function mutation of CmeR resulted in reduced fitness of *C. jejuni* in chickens and impaired ability to colonize chicks, respectively (Guo *et al*., 2008). Finally, a mutant in the *Campylobacter* bile resistance regulator (*cbrR*) gene, coding for the response regulator CbrR, was shown to be sensitive to bile components *in vitro* (Raphael *et al*., 2005). In addition, this mutant had reduced colonization ability in chicks indicating that also *in vivo* CbrR modulates resistance to bile salts in *C. jejuni*. Together, these observations indicate that bile salts and multidrug resistance is crucial for *C. jejuni* to survive in the chicken gut.

1.2.2.2 *Chemotaxis*

Since *C. jejuni* is a highly motile bacterium, chemotaxis might be an important factor promoting its migration toward favourable conditions, and thus its survival in and colonization of the intestinal mucosa. For successful chemotaxis, an intact gradient-sensing mechanism, in which adaptation has a crucial role, is indispensable. The *C. jejuni* genome contains genes encoding putative adaptation proteins: a methylesterase CheB and a methyltransferase CheR, which are both involved in a methylation-dependent chemotaxis pathway (Stephens *et al*., 2006) and together form the putative adaptation system CheBR, which is believed to be involved in the response of *C. jejuni* to environmental signals by modifying its chemoreceptors (Kanungpean *et al*., 2011). A ΔcheBR mutant was shown to have a reduced ability to colonize the chick cecum (Kanungpean *et al*., 2011). *C. jejuni* is attracted by the glycoprotein mucin, the principal constituent of mucus, and also by the bile and mucin constituent L-fucose. The amino acids aspartate, cysteine, serine and glutamate, and the salts of the organic acids citrate, fumarate, α-ketoglutarate, malate, pyruvate and succinate also act as chemoattractants (Hugdahl *et al*., 1988). Additionally, L-asparagine, formate and D-lactate were recently identified as attractants of *C. jejuni* (Vegge *et al*., 2009). Surprisingly, in this study, *C. jejuni* was not attracted to citrate and L-fucose. All these

chemicals are sensed by the transmembrane methyl-accepting chemotaxis proteins (MCP) of *C. jejuni* (Vegge *et al*., 2009). Hendrixson & DiRita (2004) identified 22 *C. jejuni* genes involved in colonization of the chicken GI tract. Severely affected colonization capacity particularly resulted from mutation in the determinant of chick colonization gene B (*docB*), encoding a putative MCP and alternatively called chemoreceptor transducer-like protein10 (Tlp10). DocC (Tlp4), another MCP, was important for obtaining wild type colonization levels. Finally, also Tlp1 is important for chick colonization since a *tlp1*-isogenic mutant showed reduced colonization ability (Hendrixson & DiRita, 2004; Hartley-Tassell *et al*., 2010). Surprisingly, all three chemoreceptors (*tlp1*, *tlp4* and *tlp10*) have been identified as being important for invasion (see further) of *C. jejuni* in chicken embryo intestinal cells (used as a model for *in vivo* invasion in chicken gut epithelial cells), but not for chemotaxis (Vegge *et al*., 2009). While it is clear that these factors contribute to *in vivo* colonization, their precise role in colonization requires further study. The putative accessory colonization factor (*acfB*), encoding a probable MCP protein, is highly upregulated in the chick cecum and although not important in the early stages of colonization, it cannot be ruled out that it might be involved in the persistence of *C. jejuni* in the chick cecum in the presence of a developed gut flora (Woodall *et al*., 2005). A number of other genes as well have been associated with *C. jejuni* chemotaxis, including the *Campylobacter* energy taxis response genes *cetA* and *cetB* (Golden & Acheson, 2002) and the chemotaxis regulatory gene *cheY*, which codes for a response regulator controlling flagellar rotation and is involved in the same signal transduction pathway as *CheBR* (Stephens *et al*., 2006). CheY shuttles between the MCP DocB and Tlp1 and the flagellar motor (Hendrixson & DiRita, 2004; Hartley-Tassell *et al*., 2010). A *cheY* mutant was affected in its colonization potential of the chick cecum (Hendrixson & DiRita, 2004). Also the production of the signal autoinducer AI-2 has been shown to be important for colonization (Quinones *et al*., 2009). Inactivation of *luxS*, the gene encoding the AI-2 biosynthesis enzyme, led to a decrease in chemotaxis toward organic acids, *in vitro* adherence to chicken hepatoma (LMH) cells and chick colonization. These observations indicate that energy taxis may be an important force in environmental navigation by *C. jejuni*, driving the organism toward optimal chemical conditions for colonization.

1.2.2.3 *Flagella and motility*

Intact and motile flagella are important colonization factors for *C. jejuni* in chickens (Nachamkin *et al*., 1993). *C. jejuni* contains one or two polar flagella. The flagellar filament consists of multimers of the protein flagellin and is attached by the hook protein FlgE to a

basal structure, embedded in the cell membrane and serving as a motor for rotation. The flagellin locus contains two adjacent genes, *flaA* (encoding the major flagellin) and *flaB* (encoding a minor flagellin). Both genes are independently transcribed, with the *flaA* gene regulated by a σ^{28} promoter and the *flaB* gene by a σ^{54} promoter (Nuijten *et al*., 1990; Hendrixson *et al*., 2001; Hendrixson & DiRita, 2004). Environmental and chemotactic stimuli modulate *flaA* and *flaB* promoter activity. Medium pH, growth temperature and the concentration of certain inorganic nutrients affect *flaB* promoter activity (Alm *et al*., 1993). Lower pH, bovine bile, deoxycholate, L-fucose, high osmolarity and chemotactic effectors such as aspartate, glutamate, citrate, fumarate, α-ketoglutarate and succinate all upregulate the *flaA* promoter. Proline, high viscosity and milk fermented by *Bifidobacterium* or *Lactobacillus* strains downregulate the *flaA* promoter (Allen & Griffiths, 2001; Ding *et al*., 2005). The *flaA* gene seems to be highly conserved among *Campylobacter* isolates and transcription is usually higher than that of *flaB* (Guerry *et al*., 1990). Transcription of σ^{54}-dependent genes, necessary for assembly of the hook-basal body filament structure, is regulated by a two-component system composed of the sensor kinase FlgS and the response regulator FlgR (Wosten *et al*., 2004). Experiments with mutants have shown that *flaA* but not *flaB* is essential for colonization of chickens (Wassenaar *et al*., 1993; Jones *et al*., 2004) although probably both are needed for full motility (Neal-McKinney *et al*., 2010). Colonization is also impaired with the mutant for the motility accessory factor 5 (*maf5*) gene, important for the formation of flagella (Karlyshev *et al*., 2002; Jones *et al*., 2004). Once *C. jejuni* reaches the cecum, it seems that mutants in the flagellar biosynthesis genes *rpoN* (encoding σ^{54}) and *fliA* (encoding σ^{28}) and the response regulator gene *flgR* could establish colonization at a high inoculation dose, albeit bacterial numbers were much lower compared to the controls and the number of chicks colonized with these mutants was extremely low (Hendrixson & DiRita, 2004; Wosten *et al*., 2004; Fernando *et al*., 2007). Chickens exposed to the *flgR* mutants showed a delayed colonization and transmission to *Campylobacter*-free chickens was not observed. However, comparable bacterial counts were detected in the ceca of birds two weeks after inoculation with either the *flgR* mutant or the wild type strain. Since bird-to-bird transmission in flocks is generally considered to be very rapid, this indicates that the FlgS/FlgR system is mainly required for initial colonization and less for survival and persistence in the cecum of chicks (Wosten *et al*., 2004). Also the *flgK* mutant, expressing only the hook, showed diminished motility and was completely attenuated for colonizing the chick cecum (Fernando *et al*., 2007). Further supporting indications that flagella are important colonization factors for *C. jejuni* in chickens was given by Hiett *et al*. (2008). These authors

demonstrated differential expression patterns of flagella proteins between a poor and a robust colonizer strain in poultry. These differentially expressed genes, coding for proteins involved in the modification of the flagellum, are located in hypervariable regions of the *C. jejuni* genome. This variability was shown to be extendable to the protein level, and thus may contribute to the survival of *C. jejuni* in its different environments and hosts.

In *C. jejuni* chicken isolates, the flagellin *O*-linked glycosylation island, responsible for successful flagellin assembly and motility, is very diverse (Karlyshev *et al*., 2005). Five genes (*cj1321 - cj1325/6*) lying in this variable region are, however, significantly prevalent among *C. jejuni* strains associated with poultry (Champion *et al*., 2005) and might therefore be important for the ability of certain *C. jejuni* strains to colonize this host. Mutagenesis and functional and structural data supported this hypothesis, with particularly *cj1324* being important for chick colonization (Howard *et al*., 2009).

The flagellar apparatus functions as a type III secretion apparatus for the *Campylobacter* invasion antigens (Cia proteins) (Konkel *et al*., 2004), important for *in vitro* cell invasion (Konkel *et al*., 1999) and chick colonization (Ziprin *et al*., 2001), and secretion is enhanced upon exposure to chicken mucus (Biswas *et al*., 2007). A correlation has been demonstrated between chicken colonization potential and *in vitro* secretion of Cia proteins (Biswas *et al*., 2007). *RpoN* mutants are completely aflagellated and as such do not secrete Cia proteins, nor do *flgK* mutants (Fernando *et al*., 2007), making it clear that the molecular basis behind the colonization mechanism in chickens is complex.

The role of motility of *C. jejuni* colonization in the chicken GI tract is not fully understood. Non-motile *C. jejuni* mutants can colonize chickens, be it at substantially reduced levels and only when chickens are inoculated with high amounts of viable cells (Wosten *et al*., 2004). Probably, motility is needed for *C. jejuni* to pass the GI tract so it can reach its protective niche, the mucus layer of the cecal crypts (Beery *et al*., 1988), and to resist gut peristalsis (Hendrixson & DiRita, 2004), hence it is important for initial colonization. It is, however, not known if motility is important in the persistence of *C. jejuni* in the intestinal tract, leading to long-term colonization. In any case, it is clear that the specialized flagellum of *C. jejuni* serves multiple functions in the adaptation of *C. jejuni* to the chicken GI tract.

1.2.2.4 *Surface-accessible carbohydrate structures and immune evasion*

Several surface-accessible carbohydrate structures (SACS) such as flagella, lipooligosaccharides (LOS), a capsule and *O*- and *N*-linked glycans contribute to *C. jejuni* colonization in chicks.

In *C. jejuni*, the lipopolysaccharide molecule only consists of lipid A and the (inner and outer) core oligosaccharide and is therefore referred to as LOS, as the high-molecular-weight *O*-polysaccharide is a capsular polysaccharide not linked to the lipopolysaccharide molecule (Karlyshev *et al*., 2000). *C. jejuni* LOS is important for host cell adhesion and invasion and chick colonization, and sialylation of the LOS outer core further enhances epithelial cell invasion (Louwen *et al*., 2008; Javed *et al*., 2012). Moreover, sialylated LOS results in reduced immunogenicity (Guerry *et al*., 2000) and increased invasion potential in Caco-2 cells (Habib *et al*., 2009). The majority of strains from human and chicken origin belonging to the clonal complex CC-21, an ecologically diverse and the largest complex in the general population structure of *C. jejuni*, were found to belong to one sialylated LOS class in particular, LOS class C, correlating with a high invasive potential (Habib *et al*., 2009). Thus, sialylation of the LOS outer core is likely to contribute to successful colonization of *C. jejuni* in a suitable host. Genes responsible for the formation of the polysaccharide capsule, surrounding the surface of *C. jejuni* cells and possibly involved in survival, adherence and evasion of the host's immune system (Roberts, 1996; Karlyshev *et al*., 2000), also play a role in colonization of the chicken intestine by *C. jejuni.* Mutation in the capsular polysaccharide transporter gene M (*kpsM*), which results in the loss of a high molecular weight glycan, and thus absence of a capsule, abolished colonization of chickens (Jones *et al*., 2004; Bacon *et al*., 2001). A *C. jejuni* mutant for the *kpsE* gene, which is unable to express any capsular polysaccharide, was not hampered in its ability to colonize the chicken intestinal tract but the number of bacteria recovered from cecum and colon were lower compared to the control (Bachtiar *et al*., 2007). Interpretation of these results is hampered by the use of different chicken *in vivo* models and bacterial strains. Capsule formation and LOS biosynthesis genes are located in hypervariable regions in the *C. jejuni* genome (Parkhill *et al*., 2000), resulting in an enormous antigenic diversity among isolates.

C. jejuni is unique in being the only known prokaryote having an *N*-linked protein modification system, which is encoded by the *pgl* multigene locus (Kakuda *et al*., 2006; Young *et al*., 2007). The *N*-linked glycosylation pathway is responsible for post-translational modification of multiple proteins, including flagellin, and is conserved among *C. jejuni* isolates (Szymanski *et al*., 1999; Karlyshev *et al*., 2005). In contrast, the only known proteins to be modified by *O*-linked glycosylation in *C. jejuni* (see above) are flagellar subunits (Karlyshev *et al*., 2005). In humans, most of the *N*-linked glycosylated proteins are highly immunogenic with their glycosyl moieties being immunodominant while only limited antibody is generated against the protein fraction (Szymanski *et al*., 1999). This indicates that

glycosylation might offer *C. jejuni* a unique system of immune evasion by masking primary amino acid sequences. A mutant in the *N*-linked general protein glycosylation pathway gene H (*pglH*) possessed an intact capsule, but was unable to glycosylate proteins and was severely reduced in its ability to colonize the chicken intestinal tract (Jones *et al*., 2004; Karlyshev *et al*., 2004). Also strains with other mutations in the *pgl* locus were affected in their ability to colonize chicks (Hendrixson & DiRita, 2004), indicating that *N*-linked glycosylation in *C. jejuni* is an important colonization determinant. However, glycan modification of Cj1496c, a glycoprotein important for *in vitro* cell invasion in human epithelial cells and initial chick colonization does not seem to influence its function (Kakuda *et al*., 2006). Moreover, most *N*-glycosylated proteins, including Cj1496c, are annotated to be periplasmatic and do not come in direct contact with host factors and the exact mechanism by which this glycosylation system contributes to colonization remains to be elucidated (Young et al., 2002; Kakuda *et al*., 2006).

To conclude, several SACS of *C. jejuni*, including the unique *N*-linked glycans, contribute to successful colonization in chicks. Not only by mediating adhesion (see further), but also by creating an enormous antigenic diversity in *C. jejuni* isolates resulting in persistent high-level gut colonization of certain strains.

1.2.2.5 *Two-component regulatory systems*

C. jejuni, like all prokaryotes, responds to environmental changes by using two-component regulatory systems (TCRSs) consisting of sensor (S) kinases and response (R) regulators, regulating *C. jejuni* gene expression (Woodall *et al*., 2005; Mikkelsen *et al*., 2011). A histidine kinase senses specific environmental triggers through autophosphorylation of the histidine residue. Subsequent transfer of the phosphate group to the corresponding response regulator turns it into an active transcription factor that can stimulate the differential expression of target genes, allowing *C. jejuni* to immediately respond to changing environmental conditions (such as several stressors, nutrients and temperature) within the chicken gut (Mikkelsen *et al*., 2011).

To date, five TCRSs have been identified in *C. jejuni* to be important for optimal chick colonization: FlgRS (Wosten *et al*., 2004) and the orphan response regulator CbrR (Stephens *et al*., 2006) (see above), the reduced ability to colonize (RacRS) system (Bras *et al*., 1999), diminished capacity to colonize (DccRS) (MacKichan *et al*., 2004) and *Campylobacter* planktonic growth regulation (CprRS) (Svensson *et al*., 2009). RacRS is responsive to temperature, and both mutation of *racR* (Bras *et al*., 1999; Woodall *et al*., 2005) or RacS

(Apel *et al*., 2012) reduce the colonization potential of *C. jejuni* (see below). DccRS controls the expression of several genes encoding probable membrane-associated proteins (Woodall *et al*., 2005). Finally, CprRS is thought to control essential biological processes, stress tolerance and biofilm formation, making it possible for *C. jejuni* to adapt to different environments (Svensson *et al*., 2009). A *ΔcprS* mutant was reported to display a dramatic dose-dependent defect for chick colonization. Thus, it is clear that the genome of *C. jejuni* harbours multiple TCRS genes, involved in all aspects of *C. jejuni* biology, which are vital for its efficient adaptation to the chicken host.

1.2.2.6 *Temperature regulation and heat shock response*

The elevated body temperature of the chicken (42°C) as compared to humans implies the transcription of many different proteins uniquely transcribed in response to the chicken GI tract environment. The RacR/RacS signal transduction system responds to temperature changes and might play an important role in chicken colonization with *C. jejuni* (Bras *et al*., 1999). Comparative analysis of the protein profile of wild type *C. jejuni* and *racR* mutants, revealed 11 proteins to belong to the RacR regulon. Three proteins were sequenced and were identified as RacR and two isoforms of a cytochrome *c* peroxidase homologue. A comparative study by Zhang *et al*. (2009) revealed 15 to 20 proteins differentially expressed by at least two-fold when *C. jejuni* was grown at 37°C or at 42°C. All identified, differentially expressed proteins are periplasmic proteins or major antigens of *C. jejuni*, or are involved in the metabolism or regulatory system. These proteins might play a role in adaptation to and pathogenicity in the different hosts of *C. jejuni*. It was demonstrated that RacR is important for growth at 42°C (Bras *et al*., 1999). DnaJ belongs to a family of heat shock proteins and plays a role in *C. jejuni* thermotolerance (Konkel *et al*., 1998). Mutation of *dnaJ* severely reduced colonization in chicks (Konkel *et al*., 1998; Ziprin *et al*., 2001). The *dnaJ* gene is located adjacent to *racR* and under the transcriptional control of RacR (Bras *et al*., 1999; Apel *et al*., 2012). While *racRS* is activated, *dnaJ* is repressed by RacR.

1.2.2.7 *Adhesion*

Campylobacter adhesion to epithelial cells of the chicken GI tract is believed to be an important step in successful colonization. Several studies contributed to the importance of intact flagella and adhesins, surface-exposed proteins, in chicken colonization. Mutation of the *Campylobacter* adhesion protein A (*capA*) gene, encoding an autotransporter lipoprotein, resulted in reduced capacity to adhere to human and chicken intestinal epithelial cells and

reduced invasion capacity in human epithelial cells (Ashgar *et al*., 2007; Flanagan *et al*., 2009). While *capA* mutation abolished colonization in a chick model in the study by Ashgar *et al*. (2007), Flanagan *et al*. (2009) did not observe reduced colonization capacity for the *capA* mutant. Moreover, since this gene is absent in many *C. jejuni* poultry isolates, the genuine contribution of *capA* to successful chick colonization is unclear (Flanagan *et al*., 2009; Friis *et al*., 2010). The *Campylobacter* adhesion to fibronectin (CadF) outer-membrane protein (OMP) was shown to bind to fibronectin, a glycoprotein of the extracellular matrix of the GI tract (Konkel *et al*., 1997), and to be important for full binding capacity of *C. jejuni* to chicken epithelial cells (Flanagan *et al*., 2009). Ziprin *et al*. (1999, 2001) demonstrated that mutants in the genes *cadF* and *pldA*, the structural gene for phospholipase A, are impaired in their ability to colonize the cecum, indicating that these genes may play a prominent role in successful colonization. However, in contrast to the highly prevalent *cadF* gene, many *C. jejuni* isolates lack the *pldA* gene (Rizal *et al*., 2010). Moreover, the biological function of *pldA* is not known, but due to its outer-membrane localization it might be involved in maintaining the functional integrity of surface exposed adhesins in some strains (Dekker, 2000). Hiett *et al*. (2008) demonstrated differential expression patterns for major OMPs in poultry between a poor and a robust colonizer strain. These differentially expressed genes are located in hypervariable regions of the *C. jejuni* genome and may contribute to the survival of *C. jejuni* in its different environments and hosts. Recently, a new adhesin, fibronectin-like protein A (FlpA), has been identified to be important for full binding capacity to chicken epithelial cells and successful colonization (Flanagan *et al*., 2009). Konkel *et al*. (2007) found that different *C. jejuni* strains compete for colonization in broilers and hypothesized that this is due to the sharing of common adhesins among these isolates and limited host epithelial cell binding places. This finding supports the hypothesis that adhesion is a key step in the colonization process of *C. jejuni* in chicks.

1.2.2.8 *Invasion*

Invasion might be an important colonization determinant of *C. jejuni* in chicks because mutations in *ciaB* as well as in the MCP genes *tlp1*, *tlp4* and *tlp10*, important for *in vitro* invasion in mammalian and chicken cells respectively (see above), severely impair cecal colonization (Ziprin *et al*., 2001; Vegge *et al*., 2009). Studies with isolated primary intestinal cells from chickens indeed showed that *C. jejuni* was able to invade chicken cells (Byrne *et al*., 2007; Li *et al*., 2008; Van Deun *et al*., 2008b), an unexpected feature since *C. jejuni* does not associate with chicken crypt epithelium *in vivo* (Byrne *et al*., 2007). Invasion capacity was

largely strain-dependent, but overall no difference was observed between isolates from poultry or human origin. Microtubule- as well as microfilament-dependent invasion was reported, which is in accordance with results obtained from invasion experiments in human epithelial cell lines (Hu & Kopecko, 1999). Many studies on the genes which are thought to play a role during invasion have been conducted on human epithelial cell lines, but thus far experiments on chicken primary epithelial cecal cells are lacking. While it is tempting to assume that invasion mechanisms in these cells are analogous to those in human cell lines, some differences do exist: *C. jejuni* can survive *in vitro* in human T84 epithelial cells by avoiding fusion with lysosomes (Watson et al., 2008), but intracellular survival seems not to be the case in the primary chicken enterocytes (Van Deun *et al*., 2008b). The lack of an immortalized chicken intestinal cell line and the complicated handling of primary chicken cecal cells clearly hamper investigation toward invasion (and other) mechanisms in chicken cecal cells. Nevertheless, the recent obtained *in vitro* and *in vivo* results described under this section suggest that invasion of *C. jejuni* in gut epithelial cells might be an important colonization determinant *in vivo*.

1.2.2.9 *Iron transport and regulation*

Regulation of the intracellular iron concentration is an important factor to secure colonization. Iron is essential for electron transfer processes and functions as a cofactor for several enzymes. It is also responsible for the generation of hydroxyl radicals. Moreover, iron availability modulates the transcription of genes belonging to several functional groups, thereby affecting the ability of *C. jejuni* to colonize the GI tract (Palyada *et al*., 2004). The soluble ferrous iron (Fe^{2+}) is readily transported across the outer-membrane via porins and is subsequently transported across the cytoplasmic membrane by a specific transporter protein, FeoB. This transporter is important for iron acquisition and intracellular survival of *C. jejuni*, as well as for successful gut colonization (Naikare *et al*., 2006). Mutants in the ferric uptake regulator (*fur*) gene, the *cfrA* gene responsible for an outer-membrane ferric enterobactin (FeEnt) receptor and the *ceuE* gene encoding a FeEnt periplasmic binding protein regulated by *fur*, are all compromised in their ability to colonize chickens, with complete absence of live bacteria for the latter two (Palyada *et al*., 2004), as were mutants in another recently identified and characterized outer-membrane FeEnt receptor CfrB, which is most prevalent in *C. coli* strains (Xu *et al*., 2010). Inactivation of *cfrB* in a *cfrA*-negative *C. jejuni* strain fully abolished its ability to utilize FeEnt as a sole iron source for growth. Moreover, the reduced colonization phenotype of the isogenic *cfrB* mutant of *C. jejuni* could not be restored by the

presence of a functional *cfrA* gene. In contrast, complementation of an isogenic *cfrA* mutant with the wild type *cfrB* gene in *trans* fully restored the ability of this *C. jejuni* mutant to utilize FeEnt. Thus, CfrB plays an important role during colonization of *Campylobacter* in chicks and cannot be compensated by other iron uptake mechanisms without affecting the colonization potential. Therefore, it is believed that CfrB is the relevant receptor for FeEnt utilization during colonization of chickens with *C. jejuni* strains producing both a functional CfrA and CfrB. Transcription levels of *chuA*, a gene believed to code for an outer-membrane receptor for hemin and hemoglobin, are increased over 40-fold in the chicken cecum, indicating that ChuA might be required for *C. jejuni* to colonize chicks (Woodall *et al*., 2005). Finally, mutation in Cj0178, a putative transferrin-bound iron utilization outer-membrane receptor, resulted in reduced colonization potential (Palyada *et al*., 2004). Given this information, it can be concluded that several iron-uptake systems are essential for the survival of *C. jejuni* and for its successful colonization in the chicken host.

Besides iron, also zinc has been reported to be an important trace element necessary for *C. jejuni* growth inside the chicken host (Davis *et al*., 2009). A *C. jejuni* mutant lacking ZnuA, the periplasmic component of a putative zinc ATP-binding cassette (ABC) transport system, had a growth defect in zinc-limiting media and was severely affected in its colonization potential in chickens.

1.2.2.10 *Oxidative and nitrosative stress defense*

C. jejuni is a microaerophilic microorganism and thus requires reduced oxygen levels for its growth. Nevertheless, it must resist oxidative stress it may encounter both in the environment and in its host, like the superoxide anion, hydrogen peroxide and biotoxic hydroxyl radicals. These stressors can result from incomplete reduction of oxygen by *C. jejuni*, or be induced by the chick immune system (Atack & Kelly, 2009). *C. jejuni* contains a wide range of enzymes involved in defense against oxidative stress. Several of these regulators have already been identified. However, the mechanism of gene regulation in *C. jejuni* is still poorly understood. Cytochrome *c* peroxidases (CcPs) are generally responsible for the conversion of hydrogen peroxide to water (Atack & Kelly, 2009). With a technique called subtractive hybridization, Ahmed *et al*. (2002) found 23 DNA sequences, including cytochrome oxidase III, to be present in a robust colonizer but absent from a poor colonizer *C. jejuni* strain. No direct link could be found that these factors correlate with the identified genes by Hendrixson & DiRita (2004), but it can be assumed that also these strain-specific genes are factors important for efficient and sustained colonization. *C. jejuni* has two CcP loci, which surprisingly do not

contribute to hydrogen peroxide resistance and thus do not protect against oxidative stress. Instead, it seems that, in *C. jejuni*, resistance to hydrogen peroxide is mainly mediated by the sole cytoplasmic catalase KatA, breaking it down to water and oxygen (Bingham-Ramos & Hendrixson, 2008; Atack & Kelly, 2009). Nevertheless, mutation in one of the two CcP loci, *docA*, located immediately upstream of *docB*, resulted in a substantial dose-dependant decrease in colonization potential (Hendrixson & DiRita, 2004; Bingham-Ramos & Hendrixson, 2008). Moreover, Woodall *et al*. (2005) found Cj0358, another putative CcP, to be upregulated 12-fold *in vivo* suggesting a role for this protein in hydrogen peroxide removal from the periplasm. By constructing an isogenic Δ*perR* mutant, deficient in the regulon of the peroxide-sensing regulator (PerR), and comparing its transcriptome profile with that of the wild type strain, Palyada *et al*. (2009) identified over 100 genes to be part of the PerR regulon. Mutation of *perR* significantly reduced *C. jejuni* motility and attenuated colonization in chickens. This study also revealed a functional network between the key players of the oxidative stress defense system, including mainly the antioxidant proteins encoded by the superoxide dismutase (*sodB*), defending *C. jejuni* against the superoxide anion, the alkyl-hydroperoxide reductase (*ahpC*) and *katA*, their transcriptional regulators *fur* and *perR* and the regulatory pathways that connect them. This indicates that there is a link between oxidative stress (PerR-regulated) and iron metabolism (Fur-regulated) in *C. jejuni.* Indeed, it was recently shown that transcription of perR is reduced in the presence of iron (Kim *et al*., 2011). Moreover, it seems that oxidative stress defense mechanisms and their proper regulation are essential for successful and efficient colonization of the chick cecum. Indeed, the colonization potential in chicks was reduced by 50,000-fold in the *C. jejuni ΔahpC* mutant, while colonization in *ΔperRΔfur*, *ΔkatA* and *ΔsodB* mutants was completely abolished. This indicates that all key players of this functional network need to be intact for successful colonization of *C. jejuni* in chicks. Garenaux *et al*. (2008) demonstrated that next to KatA, CadF and FlaA also a periplasmic protein (Cj1371) was overexpressed following exposure to paraquat, a strong oxidizing agent. In contrast, levels of SodB were unaltered, while the *Campylobacter* oxidative stress regulator CosR was downregulated by paraquat, but not by hydrogen peroxide (Hwang *et al*., 2011). CosR negatively regulates SodB and LuxS and positively regulates AhpC (Hwang *et al*., 2011). In addition, a *cj1371* mutant and a CosR knockdown were both found to be more sensitive to oxidative stress (Garenaux *et al*., 2008; Hwang *et al*., 2011). This indicates that they play a role in *C. jejuni* oxidative stress resistance and might be important for persistent chick colonization, but this has yet to be demonstrated.

The enzyme γ-glutamyl transpeptidase (GGT) is involved in maintaining cellular glutathione levels. Glutathione is an antioxidant molecule providing vital cellular protection against reactive oxygen species, generated by aerobic respiration (Tate & Meister, 1981; O'Donovan & Fernandes, 2000). GGT was shown to be present in a robust but absent from a poor colonizer *C. jejuni* strain (Ahmed *et al*., 2002), suggesting that GGT activity is not needed for initial colonization but indispensable for persistence of *C. jejuni* in the avian gut (Barnes *et al*., 2007). GGT catalyzes the conversion of gluthatione and glutamine to glutamate, and the ability of certain *C. jejuni* strains to utilize glutamine or glutathione as a sole carbon source is absolutely dependent on the presence of GGT (Hofreuter *et al*., 2008). GGT is not present in all *C. jejuni* strains (Barnes *et al*., 2007) which could explain the lower colonization capacity of strains lacking a functional GGT.

A *ppk1* and *ppk2* mutant, defective in respectively polyphosphate kinase 1 (PPK1) and 2 (PPK2), two key enzymes of the polyphosphate metabolism, were shown to have decreased invasion ability in human intestinal epithelial cells and a dose-dependent colonization defect in chicken ceca (Candon *et al*., 2007; Gangaiah *et al*., 2010). This indicates that the utilization and accumulation of polyphosphate helps *C. jejuni* to adapt to the cecal environment of the chick.

For survival and optimal colonization in the chick, *C. jejuni* must also be capable of eliciting a suitable response to cytotoxic nitric oxide (NO), a free radical produced by several cells of the host immune system that is bactericidal against *C. jejuni* (Shepherd *et al*., 2011). *C. jejuni* is protected against NO-induced nitrosative stress by NO-detoxifying mechanisms, including a nitrite reductase and its single-domain *Campylobacter* globin (Cgb) (Pittman & Kelly, 2005; Smith *et al*., 2011). Expression of Cgb in response to NO is neither regulated by Fur nor PerR, but mediated by the transcription factor NssR, regulating a nitrosative stress response regulon that also includes a truncated haemoglobin (Ctb), probably involved in oxygen metabolism (Tate & Meister, 1981; Elvers *et al*., 2005). NO detoxification in *C. jejuni* is believed to proceed via a Cgb-catalyzed dioxygenase or denitrosylase reaction, converting NO and oxygen to nitrate (Shepherd *et al*., 2011).

Many *C. jejuni* redox proteins essential for electron transfer (see further) have *N*-terminal twin-arginine translocase (TAT) signal sequences ensuring proper transport across the cytoplasmic membrane (Hitchcock *et al*., 2010). The TAT secretion system has been shown to be important for *C. jejuni* to cope with stress and for chick colonization (Rajashekara *et al*., 2009). A *C. jejuni tatC* knockout mutant had defects in biofilm formation, motility and flagellation, and was defective in survival under osmotic shock and oxidative and nutrient

stresses, impairing the efficient transmission of *C. jejuni* to a susceptible host. The *ΔtatC* mutant was unable to persistently colonize chickens which is likely the result of multiple, additive effects caused by the inability of the *tatC* mutant to translocate essential TAT substrates (Rajashekara *et al*., 2009). Also a *cj0379c* mutant, lacking a functional TAT translocated molybdo-enzyme of unknown function, was deficient in chick colonization (Hitchcock *et al*., 2010). The nitrosative stress phenotype of this mutant suggests a role for Cj0379 in the reduction of reactive nitrogen species in the periplasm.

It is clear that within its chicken host, *C. jejuni* can encounter several stressors which it must resist for successful colonization. The evidence above indicates that *C. jejuni* developed some interplaying survival mechanisms that allow the organism to cope with chicken gut-induced oxidative and nitrosative stress.

1.2.2.11 *Central intermediary and energy metabolism*

In *C. jejuni*, all enzymes necessary for a complete oxidative tricarboxylic acid cycle are present. A key step in this cycle is the oxidation of succinate to fumarate. Until recently, it was believed that in *C. jejuni* this reaction is exerted by both a fumarate reductase (Frd) and a succinate dehydrogenase (Sdh), since both enzymes were found to contribute to the total fumarate reduction of *C. jejuni in vitro* and were significantly upregulated in the chick cecum (Woodall *et al*., 2005; Weingarten *et al*., 2009). A *C. jejuni* mutant missing the intact FrdA subunit of the FrdABC enzyme was completely deficient in its succinate dehydrogenase activity *in vitro* and had reduced colonization ability in chicks. In contrast, experiments with the *sdhA* mutant of *C. jejuni* showed that Sdh exhibits no succinate dehydrogenase activity and is not required for colonization, indicating that the *sdh* operon has been misannotated. Thus, Frd is the sole succinate dehydrogenase of *C. jejuni* and is therefore essential for full host colonization (Weingarten *et al*., 2009).

To meet all of its energy demands, *C. jejuni* utilizes oxidative phosphorylation (Weerakoon *et al*., 2009). In the chicken cecum, however, *C. jejuni* encounters an environment with reduced oxygen levels to which it must elicit a suitable response to efficiently and persistently colonize this part of the gut. Microarray analysis revealed several genes involved in this response to be upregulated when *C. jejuni* enters its host compared to *in vitro* culture (Woodall *et al*., 2005), with three genes in particular: the anaerobic C_4-dicarboxylate transporter genes *dcuA* and *dcuB* as well as the aspartase gene *aspA*. Probably these genes play an important role during chick colonization. A double mutant in hydrogenase (Hyd) and formate dehydrogenase (Fdh) and a mutant in 2-oxoglutarate:acceptor oxidoreductase (OoR),

had markedly reduced colonization ability in chicks, indicating the importance of these electron donor enzymes (Weerakoon *et al*., 2009). The same authors also identified NADH:ubiquinone oxidoreductase (complex I) to play an important role because a mutant in this gene showed impaired colonization capacity. Mutants in the respiratory enzymes nitrate reductase, nitrite reductase and cbb_3-type oxidase all colonize the chicken cecum to a lesser extent (Weingarten *et al*., 2008). Moreover, these enzymes are upregulated in the chick cecum, indicating that *C. jejuni* might utilize nitrite and nitrate, as well as fumarate as a terminal electron acceptor instead of oxygen (Woodall *et al*., 2005). Especially nitrate is considered as a potential *in vivo* electron acceptor (Pittman & Kelly, 2005). It is suggested that the ability of *C. jejuni* to use gluconate as an electron donor is important for full colonization potential in the avian host (Pajaniappan *et al*., 2008). A *cj0415* mutant, lacking gluconate dehydrogenase (GADH) activity, was impaired in establishing colonization in chicks but not in mice, which can probably be explained by the higher expression level of *cj0415* at 42°C compared to 37°C (Pajaniappan *et al*., 2008).

C. jejuni is an asaccharolytic bacterium and is therefore entirely dependent on a tight set of amino acids including L-aspartate, L-glutamate, L-proline and L-serine and Krebs cycle intermediates as a primary carbon and energy source (Kelly, 2008). Mutants of the L-serine dehydratase gene *sdaA* were defective to catabolize L-serine and their colonization potential in chicks was abolished (Velayudhan et al., 2004). Moreover, *sdaA* was upregulated more than two-fold in *C. jejuni* upon colonizing the chick cecum, indicating the importance of serine for *in vivo* survival (Woodall *et al*., 2005). Also *aspA* has been demonstrated to be upregulated (by 4.8-fold) in the chick cecum (Guccione *et al*., 2008). An *aspA* mutant, which was unable to use any amino acid besides L-serine, was shown to have impaired ability to persist in the intestines of outbred chickens, which can possibly be explained by the reduced growth potential of this mutant in the avian gut, because aspartate enhances oxygen-limited growth of *C. jejuni* in an AspA-dependent way. Also mutation in *livJ*, *livK* and *cj0903c*, genes involved in amino acid transportation in *C. jejuni*, resulted in a marked colonization defect in chicks, but their precise respective biological functions are not fully understood (Hendrixson & DiRita, 2004; Ribardo & Hendrixson, 2011).

Due to an observed reduction in adhesion to and invasion in cultured epithelial cells the PEB1a protein has been regarded as a putative adhesin (Leon-Kempis *et al*., 2006; Flanagan *et al*., 2009). A *peb1A* mutant was not capable of colonizing chicks but did, however, not show a reduced binding capacity to chicken LMH cells (Flanagan *et al*., 2009). This suggests that PEB1a serves a role other than, or next to, mediating adhesion during *in vivo*

colonization. Indeed, this trans-membrane protein is believed to function as an ABC transporter of aspartate and glutamate, essential for the utilization of these amino acids as a carbon source during microaerobic growth (Leon-Kempis *et al*., 2006; Flanagan *et al*., 2009; Gangaiah *et al*., 2010). However, the two-component signal peptide of PEB1a might be responsible for its localization both in the periplasm as on the cell surface, where it could act as an adhesin (Leon-Kempis *et al*., 2006). It is unclear whether PEB1a is present in the outer-membrane, but it can definitely be found in the supernatant of *C. jejuni* cultures, indicating that the protein can be exported across the outer-membrane. Nevertheless, no direct evidence is available that PEB1a functions as an adhesin in *C. jejuni*. Therefore, the inability of the *peb1A* mutant to colonize chicks (Flanagan *et al*., 2009) is probably attributable to the inability of this mutant to utilize glutathione, glutamine and the dipeptide *γ*-glutamylcysteine, although GGT activity is not affected. Thus, GGT allows the utilization of these nutrients by generating glutamate, which is then taken up by the PEB1a-dependent transporter and subsequently used as a carbon source (Hofreuter *et al*., 2008).

To conclude, due to its assaccharolytic and microaerophylic nature, *C. jejuni* is dependent on amino acids and electron acceptors other than oxygen as primary energy sources for optimal growth. Although the underlying mechanisms are not yet fully characterized, several of the key molecules and genes of the central intermediary and energy metabolism have been identified. It is clear that disturbance in the proper metabolism of these nutrients is accompanied by a severely hampered survival potential and colonization ability in chicks.

Table 2 To date identified colonization factors of *Campylobacter jejuni* in the avian gastro-intestinal tract

Functional area	Gene name or locus	Identified/predicted protein function	Reference
Multidrug efflux pump	*cmeABC*	*Campylobacter* multidrug efflux pump	Lin *et al*., 2003
	cmeR	transcriptional repressor of *cmeABC*	Guo *et al*., 2008
	cj0561c	putative periplasmic protein	
	cbrR[*]	*Campylobacter* bile resistance orphan response regulator	Raphael *et al*., 2005
Chemotaxis	***docB***	probable methyl-accepting chemotaxis protein (MCP)	Hendrixson & DiRita, 2004
	docC	probable MCP protein	
	acfB	probable MCP protein	Woodall *et al*., 2005
	cheY	chemotaxis regulatory protein	Hendrixson & DiRita, 2004
	tlp1	chemoreceptor transducer-like protein	Hartley-Tassell *et al*., 2010
	luxS	signal autoinducer AI-2 biosynthesis enzyme	Quinones *et al*., 2009
	cheB, *cheR*	putative adaptation proteins	Kanungpean *et al*., 2011
Motility	*flaA*	major flagellin	Wassenaar *et al*., 1993; Wren & Barrow, 2004
	maf5	motility accessory factor (flagellar biosynthesis)	Jones *et al*., 2004
	rpoN	RNA polymerase σ^{54} subunit	Hendrixson & DiRita, 2004;
	fliA	RNA polymerase σ^{28} subunit	Fernando *et al*., 2007
	flgR	response regulator	Hendrixson & DiRita, 2004; Wosten *et al*., 2004

[*] Underlined and bold: genes having (probable) multiple functions

	flgK	possible flagellar hook-associated protein	Karlyshev *et al*., 2004; Fernando *et al*., 2007 Howard *et al*., 2009
	cj1321 - cj1325/6	flagellin *O*-linked glycosylation island	
Capsule formation and *N*-linked glycosylation	*kpsM*	high molecular weight glycan	Jones *et al*., 2004
	pglH	probable glycosyltransferase	Hendrixson & DiRita, 2004; Karlyshev *et al*., 2004; Wren & Barrow, 2004
	cj1496c	glycoprotein with unknown function	Kakuda *et al*., 2006
Two-component regulatory systems	***racR*** *- racS*	reduced ability to colonize regulatory system	Bras *et al*., 1999
	dccR - dccS	diminished capacity to colonize regulatory system	MacKichan *et al*., 2004
	cbrR	*Campylobacter* bile resistance orphan response regulator	Raphael *et al*., 2005
	cprR - cprS	*Campylobacter* planktonic growth regulation regulatory system	Svensson *et al*., 2009
	flgR - flgS	flagellar signal transduction system	Hendrixson & DiRita, 2004
Temperature regulation and heat shock response	*dnaJ*	heat-shock protein	Konkel *et al*., 1998; Ziprin *et al*., 2001
	racR	reduced ability to colonize response regulator	Bras *et al*., 1999
Adhesion	*capA*?	autotransporter lipoprotein	Ashgar *et al*., 2007 vs. Flanagan *et al*., 2009
	cadF	outer-membrane fibronectin-binding protein	Ziprin *et al*., 1999, 2001
	pldA	outer-membrane phospholipase A	Ziprin *et al*., 2001
	peb1A	periplasmic ABC transporter of amino acids	Leon-Kempis *et al*., 2006;
	flpA	fibronectin-like protein A	Flanagan *et al*., 2009
Invasion	*ciaB*	*Campylobacter* invasion antigen B	Ziprin *et al*., 2001

	docB	probable MCP protein	Hendrixson & DiRita, 2004;
	docC	probable MCP protein	Vegge *et al*., 2009
	tlp1	chemoreceptor transducer-like protein	Vegge *et al*., 2009; Hartley-Tassell *et al*., 2010
Iron regulation	*feoB*	specific transporter protein	Naikare *et al*., 2006
	fur	ferric uptake regulator	Palyada *et al*., 2004
	cfrA	(recessive) ferric enterobactin (FeEnt) receptor	
	ceuE	FeEnt-uptake periplasmic binding protein	
	cfrB	(dominant) FeEnt receptor	Xu *et al*., 2010
	chuA	hemin uptake outer-membrane protein	Woodall *et al*., 2005
	cj0178	putative transferrin-bound iron utilization outer-membrane receptor	Palyada *et al*., 2004
	znuA	periplasmic component of a putative zinc ABC transport system	Davis *et al*., 2009
Oxidative and nitrosative stress response	*docA*	putative cytochrome *c* peroxidase	Hendrixson & DiRita, 2004 Bingham-Ramos *et al*., 2008
	cj0358	putative cytochrome *c* peroxidase	Woodall *et al*., 2005
	sodB	superoxide dismutase	Palyada *et al*., 2009
	perR	peroxide-sensing regulon	
	ahpC	alkyl-hydroxyperoxidase (peroxide stress regulon)	
	katA	catalase	
	ggt	γ-glutamyl transpeptidase	Barnes *et al*., 2007
	ppk1	polyphosphate kinase 1	Candon *et al*., 2007
	ppk2	polyphosphate kinase 2	Gangaiah *et al*., 2010
	tatC	twin-arginine translocase (TAT) secretion system	Rajashekara *et al*., 2009
	cj0379c	TAT translocated molybdo-enzyme	Hitchcock *et al*., 2010

Central intermediary and energy metabolism	*frdABC* operon	fumarate reductase	Weingarten *et al*., 2009
	aspA	aspartae ammonia-lyase	Woodall *et al*., 2005; Guccione *et al*., 2008
	hydABCD operon	hydrogenase	Weerakoon *et al*., 2009
	fdhABCD operon	formate dehydrogenase	
	oorDABC	2-oxoglutarate:acceptor oxidoreductase	
	(12) *nuo genes*	NADH:ubiquinone oxidoreductase (complex I)	
	nrfA	nitrite reductase	Weingarten *et al*., 2008
	napAGHBLD operon	nitrate reductase	
	ccoNOQP operon	cbb_3-type cytochrome *c* oxidoreductase	
	sdaA	L-serine dehrydatase	Velayudhan *et al*., 2004; Woodall et al., 2005
	cj0415	putative oxidoreductase subunit	Pajaniappan *et al*., 2008
	livJ	putative amino acid ABC transporter periplasmic-binding protein	Hendrixson & DiRita, 2004
	livK	periplasmic-binding protein	Ribardo & Hendrixson, 2011
	cj0903c	putative amino acid transport protein	Hendrixson & DiRita, 2004
	peb1A	periplasmic ABC transporter of amino acids	Flanagan *et al*., 2009
	ggt	γ-glutamyl transpeptidase	Barnes *et al*., 2007

1.2.3 Chicken intestinal immune response against *C. jejuni*

Upon entering the chicken GI tract, *C. jejuni* is attracted to the mucus layer by chemoattractants sensed by MCPs. Generally, the host intestinal mucus layer that lines the epithelial cells prevents most commensal bacteria to make direct contact with the epithelial surface by constituting a viscous physical barrier and by harbouring secretory IgA and antimicrobial peptides (Ivanov & Littman, 2011). However, although increased viscosity has been associated with down-regulation of *flaA* promoter activity in *C. jejuni* (Allen *et al*., 2001), its modified flagellum allows the bacterium to penetrate the viscous mucus layer (Guerry, 2007) and to reach and make contact with the intestinal epithelial cells. Although *C. jejuni* is not found to be attached to chicken cecal crypt microvilli *in vivo* (Beery *et al*., 1988), the bacterium has been observed intracellular in intestinal epithelial cells of experimentally inoculated three-day-old chickens and in chicken primary cecal epithelial crypt cells *in vitro* (Van Deun *et al*., 2008b). *C. jejuni* adherence and invasion of cells of the chicken gut epithelium is initially followed by an inefficient innate immune response by the chick, eventually resulting in the production of specific antibodies (Larson *et al*., 2008; Shoaf-Sweeney *et al*., 2008). Such a response is not able to clear *C. jejuni* from the gut since most flocks that become colonized stay so until slaughter (Jacobs-Reitsma *et al*., 1995; Stern *et al*., 2001b; Stern, 2008; van Gerwe *et al*., 2009). Although not all birds in a flock were colonized, it was demonstrated that *C. jejuni* can be isolated from laying hens until an age of 42 weeks (Lindblom *et al*., 1986) and probably longer, since experimental periods exceeding one year are not documented. This implies that *C. jejuni* can evade the chicken host immune system for an extended period of time. However, reduced bacterial counts in *Campylobacter*-colonized chicks, as well as a reduction in the number of colonized chicks has been observed (Sahin *et al*., 2003; Cawthraw & Newell, 2010), indicating some mucosal clearance. In addition, with older birds it cannot be ruled out that replacement of one *C. jejuni* strain by an immunologically distinct strain (strain succession) occurred, disguising mucosal clearance of the former strain.

1.2.3.1 *Protection of young chicks against colonization*

Day-of-hatch chicks have no established gut flora and possess an immature mucosal immune system (Bar-Shira & Friedman, 2006). In the cecum, it is only after four to seven days post-hatch that an increase in cecal pro-inflammatory chemo- (such as interleukin-8 (IL-8)) and cytokine expression and heterophil numbers can be observed, upon exposure to feed and

microflora (Bar-Shira & Friedman, 2006). Hatchlings are also unprotected by adaptive immunity, which only starts to develop after a few days of life (Friedman *et al*., 2003). Nevertheless, colonization of chickens with *C. jejuni* during this critical period seems not to occur. Instead, maternally-derived antibodies generated against flagellin proteins, adhesins and other *C. jejuni* surface components are important in protecting young chickens from *C. jejuni* colonization during the first two weeks, the so called lag-phase (Sahin *et al*., 2001, 2003; Shoaf-Sweeney *et al*., 2008; Zeng *et al*., 2009). Killing of *C. jejuni* by maternal antibodies happens in a complement-mediated, strain-specific way (Young *et al*., 2007). These antibodies confer enhanced protection against challenge with a homologous strain compared to a heterologous strain (Sahin *et al*., 2003), probably because they retard motility of a homologous, but not that of a heterologous strain, as shown *in vitro* (Shoaf-Sweeney *et al*., 2008). After the lag-phase, chickens show an increased susceptibility to colonization with *C. jejuni* which coincides with a loss of maternally-derived, circulating anti-*Campylobacter* IgY antibodies, suggesting that adaptive immunity is not critical in protecting broilers from colonization (Cawthraw & Newell, 2010). Interestingly, day-of-hatch broilers have been shown to be very susceptible to *C. jejuni* colonization. Susceptibility, however, diminished again over the first few days of life (Cawthraw & Newell, 2010; Conlan *et al*., 2011). Also, transmission of *C. jejuni* between co-housed birds is lower in day-old chicks compared to two-week-old birds (Conlan *et al*., 2011). This indicates that a lack of exposure of broiler flocks to *C. jejuni* and/or reduced transmission during the early stages of rearing may also contribute to the observed lag-phase.

Developing chicken embryos have increased expression levels of several avian *β*-defensins, a group of antimicrobial peptides important in innate and adaptive immune responses that might contribute to the observed protection toward *C. jejuni* infection *in ovo* and post-hatch (Meade *et al*., 2009a). For the *β*-defensin gallinacin-6, for instance, *in vitro* antibacterial activity against *C. jejuni* has been demonstrated (van Dijk *et al*., 2007).

1.2.3.2 *Innate immune response*

The chicken intestinal innate immune system is made up of several tissues, cells (such as epithelial cells, monocytes/macrophages, dendritic cells, natural killer cells and neutrophils) and germline-encoded molecules (such as chemo- and cytokines, antimicrobial peptides and nitric oxide) that can limit both commensal and pathogenic invading bacteria (Brisbin *et al*., 2008). Some *in vitro* studies with macrophages and epithelial cells, both primary and cultured, contributed to the insight into the chicken immune response toward *C. jejuni* infection. *C.*

jejuni has been shown to be adhesive to, invasive in and to stimulate inflammatory responses from these cells (Smith *et al*., 2005; Byrne *et al*., 2007; Larson *et al*., 2008; Van Deun *et al*., 2008b). Evidence of both *in vitro* uptake of *C. jejuni* by chicken peritoneal macrophages (Myszewski & Stern, 1991) and *in vivo* presence of *C. jejuni* within chicken epithelial cells and macrophages (Ruiz-Palacios *et al*., 1991) exists.

A crucial step in the host innate immune response to bacterial entrance in the GI tract is the activation of Toll-like receptors (TLRs), expressed on a variety of cells of the GI mucosa including macrophages and epithelial cells, the latter forming the first borderline defense against invading pathogens (He *et al*., 2006; Linde *et al*., 2008). TLRs are recognized by specific bacterial ligands and, once activated, promote the expression of effector molecules such as antimicrobial peptides, NO and inflammatory cytokines. Although knowledge on avian TLR biology is only starting to unravel, very recently several chicken TLRs have been implicated to play a role in *C. jejuni* recognition. The chicken TLR4/myeloid differentiation protein-2 (chTLR4/chMD-2) complex and cell-surface expressed chTLR2 recognize *Campylobacter* LOS and lipopeptides, respectively. Both receptors are potently activated by lysed *Campylobacter* bacteria. However, loss of bacterial cell wall integrity does not seem to play a critical role in TLR activation, because also live *Campylobacter* bacteria are able to elicit a marked inflammatory response in chickens (de Zoete *et al*., 2010). TLR5 specifically recognizes conserved regions of bacterial flagellins, thereby preventing intestinal pathology. *C. jejuni*, however, lacks these TLR5-recognition sites and is therefore unable to activate chTLR5, indicating that TLR5 signaling does not play a critical role in the chick immune response against *C. jejuni* (Guerry, 2007; de Zoete *et al*., 2010). Finally, TLR21, which is unique to avian, amphibian and fish species, enables recognition of unmethylated single-stranded microbial 2′-deoxyribo(cytidine-phosphateguanosine) (CpG) DNA motifs with a broad ligand specificity. *C. jejuni* CpG DNA is internalized through endocytosis and most likely interacts with chTLR21 intracellularly, similar to the interaction of CpG DNA with the functional homologue (TLR9) in mammals (de Zoete *et al*., 2010, Keestra *et al*., 2010).

Activation of chTLR2, chTLR4 and chTLR21 results in an innate immune response through myeloid differentiation primary response gene 88 (MyD88)-dependent activation of nuclear transcription factor kappaB (NF-κB) and subsequent production of inflammatory cytokines and chemokines (Brownlie *et al*., 2009; de Zoete *et al*., 2010; Keestra *et al*., 2010). Additionally, chTLR4 and chTLR21 ligands can induce the production of inducible nitric oxide synthase-mediated NO from chicken monocytes (He *et al*., 2006). In mammals, TLR-signaling also involves a TLR4-mediated MyD88-independent pathway associated with the

induction of late phase NF-κB and interferon (IFN)-inducible genes, such as IFN-β, involved in natural killer cell activation, and maturation of dendritic cells (Yamamoto *et al*., 2004). Chickens, however, lack this pathway and therefore have an aberrant response to *C. jejuni* LOS compared to mammalian species, rendering them much more resistant to the toxic effects of these TLR4 agonists. Although the TLR4-mediated MyD88-dependent pathway, leading to early phase activation of NF-κB, is intact, this explains in part the absence of pathological signs in chicks in response to infection with *C. jejuni*, despite cell adhesion and invasion (Keestra & van Putten, 2008; Shaughnessy *et al*., 2009; de Zoete *et al*., 2010).

Upon *Campylobacter* infection, primary chick kidney cells, primary chicken embryo intestinal cells and the avian macrophage cell line HD11 express NO and pro-inflammatory cyto- (IL-6 and IL-1β) and chemokines (chIL-8) (Larson *et al*., 2008; Li *et al*., 2008b). Production of NO by activated macrophages is important for their bactericidal activity (Linde *et al*., 2008). IL-1β and IL-6 are both major mediators of the innate immune system, while IL-6 is also involved in the immunological switch from innate to adaptive immunity (Smith *et al*., 2005). IL-1β is primarily produced by monocytes/macrophages and is involved in the inflammatory response of chickens against microbial products (such as LPS) by instructing epithelial cells and macrophages to produce chemokines (Bar-Shira & Friedman, 2006). The chicken orthologue of mammalian IL-8 (CXCLi1 and CXCLi2, but here referred to as chIL-8) (Kaiser *et al*., 1999; Smith *et al*., 2005) attracts heterophils and, unlike its mammalian counterpart, also monocytes to the site of infection (Martins-Green, 2001). It has been demonstrated that the *N*-terminus of chIL-8, where the chemotactic activity resides, is structurally homologous to that of monocyte chemotactic protein-1 (Borrmann *et al*., 2007). This human chemokine is chemotactic for monocytes, probably explaining the chemotactic movement of monocytes toward chIL-8. A marked chIL-8 response is induced in chicken LMH and primary intestinal cells upon inoculation with *C. jejuni* (Brisbin *et al*., 2008; Li *et al*., 20010). Finally, also in chicken embryo intestinal cells *C. jejuni* is capable of inducing a pro-inflammatory response (Borrman *et al*., 2007; Smith *et al*., 2008).

Despite the lack of association of *C. jejuni* with chicken crypt epithelium *in vivo*, some recent reports demonstrate the initiation of a mild inflammatory response in chickens upon exposure to the bacterium. *C. jejuni* colonization in chicks after lag-phase is accompanied by infiltration of pro-inflammatory cells in mucosal tissues, although overt signs of cell invasion or pathology were not found (Larson *et al*., 2008; Smith *et al*., 2008). The number of circulating monocytes/macrophages is increased during colonization but, strikingly, heterophil numbers remain unaltered (Van Deun *et al*., 2008b; Meade *et al*., 2009b).

Expression of both TLR4 and TLR21 is readily increased in cecal tissues (Meade *et al*., 2009b; Shaughnessy *et al*., 2009) and is accompanied by a marked *chIL-8* induction (Smith *et al*., 2008; Shaughnessy *et al*., 2009) and moderately induced *IL-1β* and *IL-6* expression levels (Keestra & van Putten, 2008; Smith *et al*., 2008). Because the intestinal bacterial load in *Campylobacter*-colonized chicks does not lower, there clearly exist some mechanisms that are responsible for controlling this pro-inflammatory response (Smith *et al*., 2008). Expression levels of anti-inflammatory *IL-10*, *IL-13* and transforming-growth factor *β*4 (*TGF-β4*) were not detected in cecum, ileum and spleen, and the signals modulating the pro-inflammatory response, resulting in sustained and unaffected *C. jejuni* colonization, are yet unknown (Smith *et al*., 2008; Shaughnessy *et al*., 2009). *C. jejuni* colonization in chicks significantly reduces expression levels of several antimicrobial peptide genes (Meade *et al*., 2009a). This downregulation might represent one mechanism whereby *C. jejuni* modulates the immune response, limiting the efficacy of these antimicrobial factors and enabling itself to persistently colonize its host at high levels. In a recent study by Shaughnessy *et al*. (2011) 270 genes were found to be significantly differentially expressed after 20 h in four-week-old chicks colonized with *C. jejuni* compared to *C. jejuni*-free chicks. These genes corresponded to the activation of several biological processes, including immue responses. Although differences in expression were only marginal, this response was hypothesized to point toward an innate T cell response in the ceca of chickens 20 h after inoculation with *C. jejuni* (Shaughnessy *et al*., 2011).

1.2.3.3 *Adaptive immune response*

The type of immune response generated against *C. jejuni* depends on the cytokine microenvironment induced by the chick innate defense cells. This in turn is determined by the interaction of TLRs and other pathogen recognition receptors expressed on these cells with their respective ligands. In chickens, not all of these receptors and cytokines are fully identified yet, making the switch from innate to adaptive immunity in this species not completely understood (Brisbin *et al*., 2008).

In chickens, intestinal antigens are capable of entering the bursa of Fabricius, the site of primary B cell development (Brisbin *et al*., 2008). Chickens have an incomplete antibody (IgM and IgY) response toward T cell-independent type 2 antigens. which activate B cells independently of T cells (Jeurissen *et al*., 1998). Because these antigens are usually of polysaccharide nature, an insufficient humoral response toward certain SACS of *C. jejuni* might contribute to the inability of the chicken immune system to clear this microorganism,

despite the antigenic potential of *C. jejuni* LOS and its capsule (Oza *et al*., 2002) and the marked immunogenicity of *C. jejuni* flagellin (Widders *et al*., 1998). Moreover, an OMP extract of *C. jejuni* has been shown to cause apoptosis of chicken blood and spleen lymphocytes, probably promoting immune evasion of *C. jejuni* in the chick (Zhu *et al*., 1999). An antibody response to *C. jejuni* might, however, contribute to protection against intestinal colonization of chickens, which show a significant increase in specific mucosal and circulating IgG (IgY) and IgA and circulating IgM antibody titres when colonized with *Campylobacter* (Cawthraw *et al*., 1994; Widders *et al*., 1998). In these studies, flagellin was shown to be the immunodominant antigen, which is rather peculiar due to the lack of functional TLR5-recognition sites in *C. jejuni* flagellin, permitting TLR5 evasion (Guerry, 2001; de Zoete *et al*., 2010). Nevertheless, vaccinating chicks with a hybrid protein based on *C. jejuni* FlaA induced a specific response against this protein and reduced colonization in these birds (Khoury & Meinersmann, 1995). An antibody response specific for native flagellin was also induced in the serum of chickens immunized with purified *C. jejuni* flagellin. Serum and GI secretion antibodies specific for *C. jejuni* whole cells were, however, only induced when flagellin was complemented with killed *C. jejuni* whole cells, resulting in reduced cecal *C. jejuni* counts in these birds (Widders *et al*., 1998). This might indicate that the epitopes of *C. jejuni* flagella are not accessible for these antibodies in intact bacteria and that possibly other antigens, not detected in this study, were responsible for the induction of anti-*C. jejuni* antibodies reducing the cecal bacterial load. Recent studies gave more insight into this enigma and identified additional immunogens of *C. jejuni* promoting the humoral immune response in chicks. Amongst others CfrA, CmeC, Cj0091 (belonging to a lipoprotein-encoding operon), the lipoproteins CjaA and CjaC (mediating amino acid transport), CadF and LOS were shown to be immunogenic and expressed during *in vivo* colonization (Shoaf-Sweeney *et al*., 2008; Zeng *et al*., 2009; Oakland *et al*., 2011). Both the sera of young chicks free of *C. jejuni* and older birds colonized with the bacterium were reactive against recombinant CfrA, indicating that they are not only passed from the mature hen to the hatchling but are also induced during colonization of broilers after the lag-phase (Zeng *et al*., 2009).

Intestinal epithelial cells might contribute to a mucosal IgA response by the GALT, located beneath the epithelial cell border in the lamina propria, in a T cell-dependent manner by producing IL-6 after contact with *C. jejuni* (Fagarasan, 2008). Secretory IgA is generally responsible for preventing sub-epithelial translocation of commensal bacteria by preventing their adhesion to epithelial cells or returning bacteria that already reached the basolateral site,

without eliciting an inflammatory response (Brisbin *et al*., 2008). Moreover, by its resistance to normal intestinal proteases, through dimerization on the surface of mucosal epithelial cells, IgA is ideally suited for host defenses at the mucosal surface of the GI tract (Phalipon *et al*., 2002). IgA might thus play an important role in limiting the mucosal immune response to *C. jejuni* in chickens and redirecting it toward tolerance.

Most *C. jejuni* strains possess genes encoding a cell death-promoting cytolethal distending toxin (CDT) of which the expression is induced in both the avian and human gut (Abuoun *et al*., 2005). During human infection with *C. jejuni*, neutralizing antibodies against CDT are induced but not during colonization in chickens. It seems that production of this toxin in general is not important for chick colonization as opposed to its suspected role during pathogenesis in humans (Abuoun *et al*., 2005; Biswas *et al*., 2006).

As mentioned earlier, genetically distinct chicken lines may differ in their susceptibility toward cecal *C. jejuni* colonization (Stern *et al*., 1990a). Further research in this area revealed insulin receptor signaling and metabolism process pathways to be key players of this differential response (Li *et al*., 2010). In a more resistant line, lymphocyte activation, lymphoid organ development functions and circadian rhythm were important in the cecal host defense upon *C. jejuni* inoculation. In a more susceptible line, cell differentiation, communication and signaling pathways were important during host defense, with a marked upregulation in lipid, glucose and amino acid metabolism.

1.2.3.4 *Chicken systemic immune response to C. jejuni*

Commensal bacteria in general do not colonize outside the GI tract, but strangely enough *C. jejuni* can readily be found in various extraintestinal organs of broilers too. Up to seven days after oral and cloacal inoculation, the bacterium was found in the thymus, spleen, liver/gallbladder and bursa of Fabricius (Cox *et al*., 2005; Van Deun *et al*., 2008b; Meade *et al*., 2009b). In a study with two-week-old chicks that were inoculated with *C. jejuni* at day-of hatch, high bacterial numbers (> 5 log cfu/g) were isolated from spleen and liver of most of the birds (Lamb-Rosteski *et al*., 2008). In addition, *C. jejuni* was isolated from the reproductive tract and ovarian follicles of laying hens (Cox *et al*., 2009). The dissemination of *C. jejuni* to other organs seems to be correlated with the invasive potential in primary cecal epithelial cells of chicks (Van Deun *et al*., 2008b), suggesting that *C. jejuni* translocates the epithelial barrier transcellularly (through the chicken crypt epithelium) rather than paracellularly (between cells).

This frequently observed systemic colonization indicates that *C. jejuni*, despite the induction of secretory IgA by the GALT, is capable of breaching the gut epithelial barrier. As in the GI tract, this happens without developing pathology or inducing excessive inflammation, although chicks can mount an adaptive T cell response to *C. jejuni* when it reaches and colonizes the liver (Jennings *et al*., 2011). In colonized flocks, almost all birds carry *C. jejuni* in their ceca but significantly less birds harbour the bacteria in their liver tissues (Jennings *et al*., 2011). Whether host-specific differences decide over *C. jejuni* dissemination, or a T cell response is responsible for the eradication of *C. jejuni* from the host liver in some animals, is not known. In any case, *C. jejuni*-specific antibody responses are apparently not capable of clearing the bacterium from the chicken gut, but nevertheless do indicate that there indeed must have been a preceding close interaction between *C. jejuni* and the host epithelial cells.
The two chicken lines used in the study of Li *et al.* (2010) also differed in their systemic response to *C. jejuni* (Li *et al*., 2011). In the spleen, a secondary lymphoid organ of the avian immune system important for lymphocyte activation, proliferation and differentiation, the response to *C. jejuni* in the more resistant line was characterized, as in the cecum, by lymphocyte activation and differentiation. In addition, splenic host genes for humoral responses and Ig heavy and light chain were upregulated. These responses initiate adaptive immune responses to *C. jejuni* and are probably responsible for an increased genetic resistance to systemic *C. jejuni* colonization. In the susceptible line, genes for regulation of erythrocyte differentiation, hematopoiesis and RNA biosynthesis processes were downregulated. This study also revealed distinct innate defense mechanisms against *C. jejuni* by the two chicken lines. Apoptosis and cytochrome *c* release from mitochondria was associated with increased resistance against *C. jejuni* colonization. Probably, these events induce increased apoptosis of infected host cells, thereby destroying the habitat of the bacteria and contributing to the increased resistance to splenic colonization with *C. jejuni*.

1.2.4 Interaction with the host microbiota

Little is known currently about the effect of the natural avian gut microbiota on the level of *C. jejuni* colonization. In general, host microbiota imposes a colonization barrier for intruding pathogens by competing for nutrients and host receptors. Their composition, however, can alter the outcome of invading enteric bacteria (by e.g. altering the virulence properties of these bacteria), resulting in either clearance or colonization (Keeney & Finlay, 2011). Although it has been suggested that the colonization pattern of *C. jejuni* in chicks is mainly

determined by the chicken host and not by the host microbiota (Ridley *et al*., 2008b), also the composition of the latter might contribute to the observed colonization pattern. Changes in *C. jejuni* loads in the commercial turkey intestine seemed to correlate to, but were not dependent on, two acute transitions in the cecal microbiota composition during the turkey development phases (Scupham, 2009). With an approach called antibiotic dissection, day-old turkey poults were inoculated with cecal contents of *Campylobacter*-free adult turkeys after which the microbial communities in these poults were modified by different antibiotic treatments. Molecular examination of the constituents of these communities detected that a subtype I of *Megamonas hypermegale* correlated with decreased colonization ability of *C. jejuni*, while a virginiamycin-derived cecal microbiota seemed to be correlated with enhanced colonization ability (Scupham *et al*., 2010). These results indicate that *C. jejuni* may respond to the presence of specific subsets of the avian gut microbiota. It has, however, to be examined if the effect of these gut microbiota alterations on *C. jejuni* in turkeys also applies to chicks.

1.2.5 Hypothetical mechanism of the interaction between *C. jejuni* and the chicken gut

The interaction of *C. jejuni* with its chicken host is very complex, evidenced by the extensive interplay between several key mediators important for successful and persistent colonization of the chicken GI tract. Clearly, this dual interaction is influenced by both the *C. jejuni* strain and the chicken line involved. Despite the lack of a developed pathology, a pro-inflammatory response is developed in the chicken intestinal mucosa during asymptomatic colonization with *C. jejuni*, suggesting a significant interaction between the bacteria and the epithelial cell layer of the chicken gut.

Upon entering the chicken GI tract, *C. jejuni* moves toward the intestinal epithelial border, mediated by chemotaxis. *C. jejuni* is attracted by intestinal mucins, as well as several amino acids, carbohydrates and salts of organic acids, while the chemoattractive properties of L-fucose are controversial (Vegge *et al*., 2009). *C. jejuni* responds to these chemicals via methyl-accepting chemotaxis proteins (Vegge *et al*., 2009). For moving toward the most favourable conditions for growth, *C. jejuni* needs intact flagella and it seems that especially *flaA*, *flgK*, *cj1324* and *maf5* are crucial for colonizing the chicken gut (see 1.2.2.3). Upon reaching the chicken gut epithelial boarder it is believed that *C. jejuni* adheres to the epithelial cells, mediated by intact flagella and surface-exposed proteins. In particular CadF and FlpA were identified as important adhesins, while the potential contribution of CapA is less clear (see 1.2.2.7). Also several surface-accessible carbohydrate structures of *C. jejuni*, such as

LOS and an intact capsule (see 1.2.2.4) are involved in adhesion, with in particular *kpsM* and *pglH* being important for colonization of the chicken intestinal tract. Adhesion of *C. jejuni* to gut epithelial cells may be followed by marginal invasion in these cells. Upon exposure to chicken mucus, the flagellar apparatus increases the secretion of Cia proteins. Also *C. jejuni* LOS is important for epithelial cell invasion and sialylation of the LOS outer core enhances this trait (Louwen *et al*., 2008; Habib *et al*., 2009). *C. jejuni* is not able to survive for long periods in primary chicken cecal epithelial cells (Van Deun *et al*., 2008b). Therefore, intracellular replication in these cells is probably not important for persistent *in vivo* colonization. Rather, invasion of cecal crypt epithelial cells would be followed by evading these cells allowing *C. jejuni* to replicate in the mucus, which seems to provide all necessary nutrients for optimal growth, and re-invasion to escape mucosal clearance (Van Deun *et al*., 2008b). Strangely, the invasion capacity of *C. jejuni* in primary chicken cecal epithelial cells *in vitro* is not correlated with *in vivo* gut colonization (Van Deun *et al*., 2008b). Therefore, the genuine contribution of epithelial cell invasion during cecal colonization of chicks with *C. jejuni* is not clearly definable and can only be speculated on.

C. jejuni entrance in the avian GI tract induces an early production of chIL-8 by intestinal epithelial cells, followed by macrophage recruitment and production of pro-inflammatory cytokines. This is, however, not accompanied by the recruitment of heterophils (the avian equivalent of mammalian neutrophils) to the site of infection. In a later stage, a specific mucosal IgA response is mounted against *C. jejuni*, but this induction is not capable of clearing the bacterium from the gut. This humoral response is moreover not able to prevent *C. jejuni* from further interacting with and translocating across the gut epithelium and to disseminate systemically. Also the specific T cell response that is triggered upon *C. jejuni* entrance in the extra-intestinal organs does not result in clearance from these tissues, or pathology. *C. jejuni* is thus capable of evading the inefficient host immune response and the chicken host might be involved in maintaining homeostasis during persistent colonization (see 1.2.6).

In **figure 1**, a schematic overview is given of a simplified hypothetical mechanism involved in the interaction of *C. jejuni* with the chicken gut, after lag-phase, leading to successful and persistent colonization of the GI tract, without developing pathology.

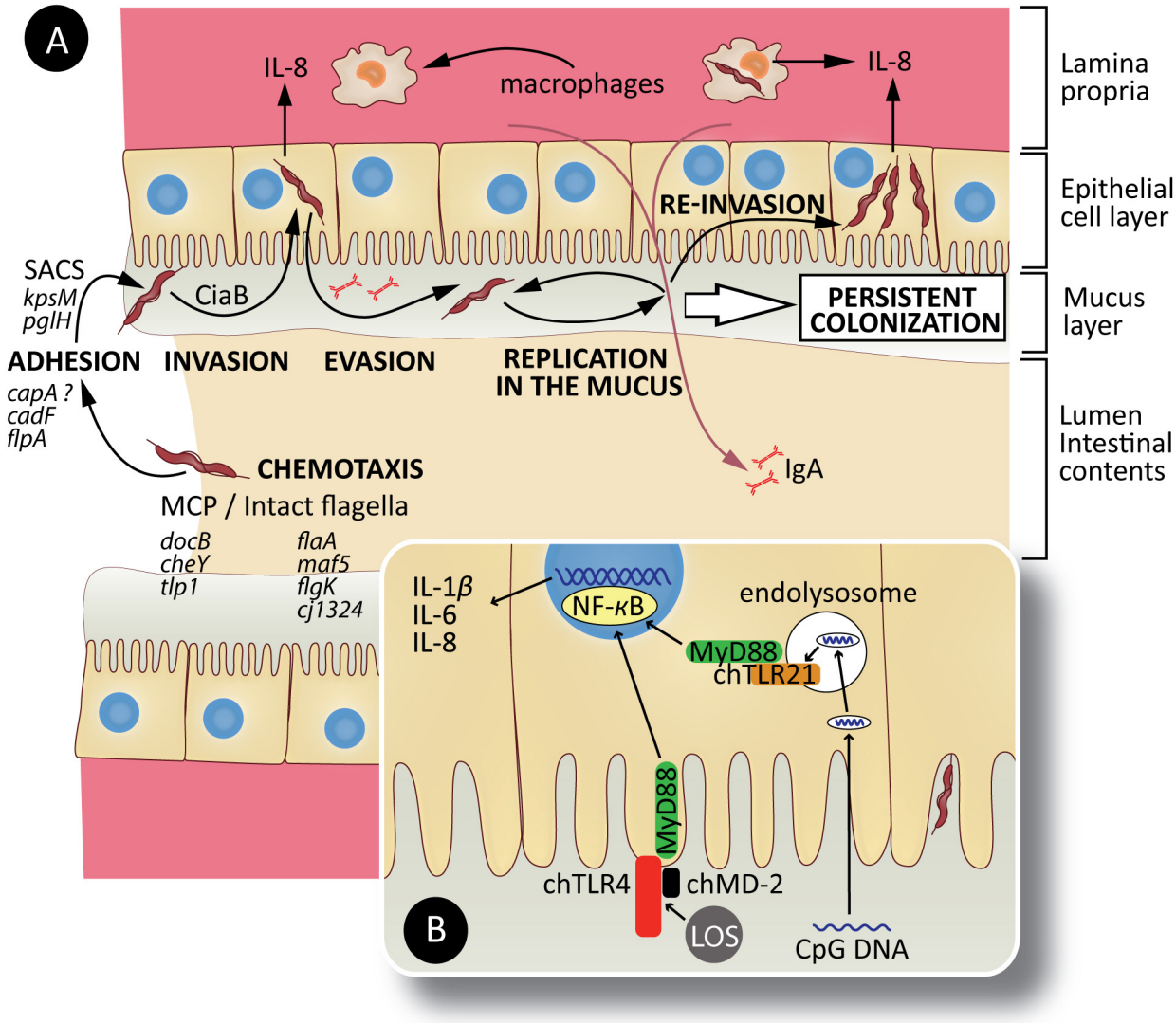

Figure 1 Intestinal *Campylobacter jejuni*-chicken crosstalk leading to persistent colonization. (A) Colonization mechanism. Upon entering the chicken gastro-intestinal (GI) tract, *C. jejuni* is attracted to the mucus layer by chemoattractants sensed by methyl-accepting chemotaxis proteins (MCP). Intact flagella are critical for this movement and allow *C. jejuni* to further migrate toward the epithelial barrier where several of its surface-accessible carbohydrate structures (SACS) modulate adhesion to and invasion of epithelial cells. Also mucus modulates invasion, by inducing the secretion of *Campylobacter* invasion antigen B (CiaB). An invasion-evasion cycle followed by replication in the mucus is probably responsible for the high numbers at which *C jejuni* colonizes the GI tract. The chick immediately responds to *C jejuni* entry by chIL-8 production from epithelial cells and recruitment of macrophages. Several other mechanisms such as stress responses are further essential for both initial and persistent colonization of *C jejuni* in the chicken GI tract. Long-term colonization is followed by the induction of specific mucosal immunoglobulin A (IgA), but *C. jejuni* translocation across the epithelial barrier toward the lamina propria, possibly by a transcellular route, is not prevented. (B) Intracellular inflammatory response. Expression levels of chicken Toll-like receptor 4 (chTLR4) and 21 (chTLR21) are increased in both epithelial cells and macrophages, which produce nuclear factor-κB (NF-κB)-mediated cytokines (IL-1β, IL-6, chIL-8) after binding their specific ligands, by triggering the myeloid differentiation primary response gene 88 (MyD88)-dependent pathway. *C jejuni* lipooligosaccharide (LOS) is sensed by the surface-exposed complex chTLR4/ch myeloid differentiation protein-2 (MD-2), while *C jejuni* 2′-deoxyribo(cytidine-phosphateguanosine) (CpG) DNA is endocytosed and recognized by intracellular TLR21. This controlled inflammatory response is neither followed by pathological signs, nor clearance of the bacterium from the GI tract.

1.2.6 Commensal *C. jejuni* colonization in chicks: immunological tolerance?

In mammals, commensal infections are characterized by the absence of a neutrophil infiltrate or a classical inflammation as seen during pathogenic infection (MacPherson & Uhr, 2004), indicating that the interaction between *C. jejuni* and its chicken host is indeed of commensal nature. Intestinal homeostasis during commensal colonization requires that a pro-inflammatory response is rapidly controlled. In mammals, not much is known about the host regulatory mechanisms that contribute to tolerance without reducing bacterial numbers, but, restricting the bacteria to the lumen (so they cannot reach the epithelial cells and the immune system) and inducing an anti-inflammatory response are believed to induce a state of "immunological ignorance" (Ivanov & Littman, 2011). Due to a lack of knowledge about the interaction between *C. jejuni* and the chicken immune system, it remains unclear how homeostasis is maintained in chickens colonized with *C. jejuni*. An apparent induction of a mild intestinal pro-inflammatory response, the inability to demonstrate upregulation of anti-inflammatory cytokines, occasional invasion of cecal crypt epithelial cells and regular dissemination to extra-intestinal organs upon *C. jejuni* colonization of the chicken host, suggests that their interaction is not a tale of ignorance but rather a cohort of active processes, exerted by the two partners, resulting in "immunological tolerance". *C. jejuni* itself might escape or alter the inflammatory response by, for instance, down-regulating antimicrobial peptide gene expression in the chicken gut, but other potential mechanism(s) or bacterial factor(s) of *C. jejuni* involved in immune evasion are currently not known. Alternatively, the chicken host might support tolerance to maintain homeostasis during persistent, asymptomatic colonization (Pédron & Sansonetti, 2004).

First of all, the differential composition of the chicken intestinal mucus layer, compared to its human counterpart, probably plays an important role in promoting homeostasis during *C. jejuni* colonization. Chicken intestinal mucins have been shown to reduce the adhesive and especially the invasive capacity of *C. jejuni* in human primary and cultured intestinal epithelial cells (Byrne *et al*., 2007; Alemka *et al*., 2010). In contrast, human-derived mucus promotes adhesion and entrance (Byrne *et al*., 2007). Moreover, MUC2, the most abundantly secreted mucin in the human intestine, is a major chemoattractant for *C. jejuni* and induces the expression of several colonization- and virulence-associated genes (Tu *et al*., 2008). To date, no such properties have been assigned to chicken mucins. Host intestinal mucins can be either secreted or expressed at the apical surface of the (cecal) mucosal epithelial cells and are readily found to be coated with fucosylated glycans in terminal positions (Stahl *et al*., 2011).

Although the chemotactic properties of L-fucose were not validated by Vegge *et al*. (2009), it is believed that *C. jejuni* is attracted to, and binds with both mucin and L-fucose. Presence of the latter at certain concentrations might moreover increase *C. jejuni flaA* promoter activity (Allen *et al*., 2001). Therefore, fucosylated glycans may function as adherence factors for *C. jejuni*. In addition, although it was believed until now that *C. jejuni* is an asaccharolytic organism, very recent evidence indicates that some strains are able to utilize L-fucose as a substrate for growth (Stahl *et al*., 2011). Thus, chemotaxis toward, adhesion to and subsequent utilization of L-fucose by *C. jejuni* strains possessing a functional L-fucose uptake and metabolism pathway provides them with a competitive advantage. This seems, however, to be only the case during pathogenic (human), but not during commensal (chick) colonization (Stahl *et al*., 2011), were chemotaxis toward amino acids is believed to be less effective due to the higher body temperature (Baserisalehi & Bahador, 2011). Probably, next to decreasing the intestinal barrier permeability to *C. jejuni*, the highly sulfated fucosylated *O*-glycan mucin structures found in chickens decrease the accessibility of, and thus the responsiveness of *C. jejuni* to L-fucose. Indeed, upon feeding young chicks with an excess of free L-fucose also here a competitive colonization advantage was observed for wild-type *C. jejuni* over a mutant lacking a functional fucose permease gene, important for L-fucose transport into the bacterial cell (Stahl *et al*., 2011). Thus, a high degree of L-fucose masking through increased sulfation might give further explanation to the lack of association of *C. jejuni* with the chicken crypt epithelium *in vivo*. To conclude, there is increasing evidence that the composition of the chicken mucus layer is involved in the hindered contact between *C. jejuni* with the chicken intestinal epithelial surface. Indeed, *C. jejuni* is not closely associated with chicken crypt epithelium *in vivo* but rather resides in the mucus within the lumen of the crypts (Beery *et al*., 1988). However, the effect of chicken mucus on *C. jejuni* invasion in primary chicken epithelial cells has not yet been examined. Moreover, as the bacterium can be frequently detected in extra-intestinal organs of chicks, the mucus layer is not likely to be an efficient barrier to prevent close interaction with *C. jejuni* and the intestinal epithelial lining. In contrast, it seems that it indirectly promotes *C. jejuni* invasion through the secretion of Cia proteins (Biswas *et al*., 2007). Further research will therefore have to reveal the genuine contribution of the mucus layer to GI and systemic colonization of *C. jejuni* in chicks.

Also the adaptive immune system of the chick might participate in the tolerogenic response to *C. jejuni*. Upon intestinal colonization, specific IgA against *C. jejuni* is induced. IgA is believed to induce the modulation of epitope expression by bacteria and to reduce intestinal pro-inflammatory signaling (Peterson *et al*., 2007). This indicates that the induction of IgA

could lead to immune evasion, but whether the induction of IgA in chickens colonized with *C. jejuni* might be responsible for the non-inflammatory *C. jejuni*-chicken gut relationship is not clear.

Next, murine intestinal epithelial cells are tolerized to LPS early after birth by exposure to exogenous LPS, facilitating microbial colonization and the establishment of a stable intestinal host-microbe homeostasis (MacPherson & Uhr, 2004). Whether in chickens LOS tolerance in the gut is involved in a tolerance-oriented integrated mucosal immune system, allowing commensal colonization of *C. jejuni*, is not clear.

Finally, chickens have an aberrant response to *C. jejuni* LOS and flagellin, due to the absence of a late phase NF-κB response and TLR5 recognition sites, respectively. Only the first is likely to contribute to the differential *C. jejuni* response in humans and chicks because *C. jejuni* escapes TLR5 recognition in humans too (de Zoete *et al.*, 2010). Next to these responses, colonized chickens might further induce tolerance by expressing factors that blunt *C. jejuni* components which could induce inflammation (MacPherson & Uhr, 2004). However, potential candidates have not yet been identified.

1.2.7 Conclusion

Upon entering the chicken GI tract, *C. jejuni* establishes a complex interaction with its host, resulting in persistent high-level cecal colonization. This inefficient, controlled inflammatory response is not capable of clearing *C. jejuni* from the chicken gut and many processes might be involved in redirecting the response toward tolerance. The underlying mechanisms of the crosstalk between *C. jejuni* and chicks are just now starting to be unravelled and further research is warranted. Especially the mechanisms allowing this bacterium to persistently evade the immune response should deserve full attention. After all, a better understanding of the chick immune response upon *C. jejuni* entrance, as well as further elucidation of the colonization mechanism of the bacterium in this host might promote the development of effective control measures to clear this human pathogen from poultry lines. For this purpose, it might be of particular interest to identify chicken factors, if any, involved in blunting *C. jejuni* virulence factors, while *C. jejuni* colonization factors identified to date might hold promise for effective subunit vaccines. Moreover, the differential disease outcome in chicks and humans upon exposure to *C. jejuni* needs to be explained. Could it be due to the differences in mucin composition, TLR signaling, effect of CDT or humoral responses in these hosts, or are there other, yet to define, mechanisms that determine the commensal or

pathogenic nature of *C. jejuni*. Answering these questions could explain why and how a single bacterium is capable of causing severe inflammatory disease in one host while being (seemingly?) completely harmless in another.

Besides genes which are probably necessary during colonization of the GI tract in a wide range of animal species in general, it seems that *C. jejuni* needs a distinct set of gene products for optimal adaptation to the chicken intestinal environment, resulting in high-level cecal colonization. However, the mechanisms by which these factors interplay to form the basis behind the complex interaction of *C. jejuni* with its avian host remain largely unclear. The incomplete understanding of the dual interaction between *C. jejuni* and the chick immune system is at least in part responsible for the lack of effective control measures (see 1.3).

1.3 *CAMPYLOBACTER* CONTROL IN POULTRY

In the past few years, several quantitative risk assessments for *Campylobacter* in poultry meat have been developed as a guidance tool to control the presence of this zoonotic pathogen throughout the poultry meat production chain (Nauta *et al*., 2009). Although there is considerable variation between countries in the approach of these models, all risk assessments conclude that aiming to reduce the *Campylobacter* levels on broiler carcasses after evisceration is the most effective intervention measure, rather than reducing its prevalence. Besides reducing external surface contamination of broiler carcasses from *Campylobacter*-colonized flocks directly, by physical or chemical means (Rosenquist *et al*., 2006; Boysen & Rosenquist, 2009), reduced *Campylobacter* numbers on carcasses can also be obtained indirectly. On-farm intervention measures aime to prevent *Campylobacter* introduction and transmission in poultry flocks or to reduce intestinal *Campylobacter* counts in colonized animals leading to reduced contamination levels of the carcasses of these animals after processing. Moreover, because the intestine of living poultry is the only amplification site for *Campylobacter* throughout the entire food chain, reducing the cecal *Campylobacter* load in poultry during primary production is expected to significantly reduce the incidence of human campylobacteriosis (Lin, 2009).

In Denmark, a quantitative microbial risk assessment of human campylobacteriosis associated with thermotolerant *Campylobacter* spp. in broiler chickens was developed. The simulations showed that reducing the number of *Campylobacter* bacteria on chicken carcasses by 2 logs

causes a 30-fold reduction in the incidence of campylobacteriosis in humans (Rosenquist *et al*., 2003). Bacterial counts in cecal contents of *Campylobacter*-colonized broiler flocks should be reduced by roughly one log more (i.e. 3 logs) to obtain a similar reduction in the number of human campylobacteriosis cases (Reich *et al*., 2008). A Belgian risk assessment showed that the incidence in Belgium would be reduced by 48%, 85% and 96% when respectively a one log, two log or three log reduction of the *Campylobacter* contamination on carcasses would be achieved (Messens *et al*., 2007).

Theoretically, controlling *Campylobacter* colonization in poultry on-farm may be achieved in a number of different ways, including hygienic and biosecurity measures (1.3.1), water treatment (1.3.2), supplementing plant-derived additives to the feed (1.3.3), bacteriophage application (1.3.4), vaccination (1.3.5), passive immunization (1.3.6) and application of competitive exclusion microflora, probiotics and prebiotics (1.3.7) or bacteriocins (1.3.8). It is important to differentiate between prevention and colonization-reducing measures, which intervene at a different stage of the colonization process. Preventive measures, summarized in **table 2**, aim at reducing the probability of birds to become colonized with *Campylobacter*, while colonization-reducing measures, presented in **table 3**, strive for a reduced cecal *Campylobacter* load in colonized birds prior to slaughter, thereby reducing surface contamination of the carcasses. Moreover, also by improving health and welfare of the animals colonization might be reduced (Bull *et al*., 2008). Finally, genetic selection could also contribute in combating *Campylobacter* colonization in poultry (Kapperud *et al*., 1993), when poultry lines with improved overall immunological responsiveness, being more resistant to colonization with this pathogen, are developed (Swaggerty *et al.*, 2009). Some antibiotics efficiently reduce *C. jejuni* counts in the broiler chick GI tract (Farnell *et al*., 2005), but their use is controversial due to concerns on development of antibiotic resistance in *C. jejuni*, which may compromise treatment of human campylobacteriosis (Dibner & Richards, 2005; Zhu *et al*., 2006).

Table 1 Preventive on-farm control measures to reduce *Campylobacter* prevalence and transmission in poultry flocks.

Intervention measure	Intervening stage in colonization process	Points of attention	References
Hygiene and biosecurity farming practices	Reduced prevalence of *Campylobacter*-colonized flocks	Need to be properly applied; not enough as a single intervention measure due to constant contamination pressure	van de Giessen *et al*., 1998 Gibbens *et al*., 2001 Hald *et al*., 2007 Rosenquist *et al*., 2009
Drinking water treatment	Reduced transmission as well as reduced crop and pre-chill carcass contamination by adding organic acids Reduced risk for *Campylobacter* colonization when chlorinating drinking water	Only a limited effect on *Campylobacter* prevalence in broiler chickens No reduced *Campylobacter* prevalence observed under commercial settings	Chaveerach *et al*., 2002 Chaveerach *et al*., 2004b Ellis-Iversen *et al*., 2009 Stern *et al*., 2002
Feed additives	Reduced crop contamination and reduced probability of chickens to become colonized when organic acids are supplemented to the feed	Contradictory observations	Heres *et al.*, 2004 Van Deun *et al*., 2008a Solis de los Santos *et al*., 2008 Skanseng *et al*., 2010 van Gerwe *et al*., 2010
Vaccination	Increased resistance to colonization with *Campylobacter* upon treating with killed whole cells and subunit vaccines	Incomplete understanding of the chicken immune system hampers development of an effective vaccine; genomic instability of *C. jejuni*?	Widders *et al*., 1996 Rice *et al*., 1997 Widders *et al*., 1998 Khoury & Meinersmann, 1995 Wyszynska *et al*., 2004 Buckley *et al*., 2010
	No *Campylobacter* could be recovered from ileum with subunit (Omp18/CjaD) *Salmonella*-vectored vaccine	Cecal *Campylobacter* counts were not determined	Layton *et al*. 2010
Passive immunization	Substantial reduction (>99%) in fecal *C. jejuni* counts upon treating with Immunoglobulin preparations	Optimization and experimental trials in industrial or farm settings are needed to prove efficacy	Stern *et al*., 1990b Tsubokura *et al*., 1997
Competitive exclusion microflora	Increased resistance to *Campylobacter* colonization with feeding animals (un-)defined microbiota	Contradictory observations	Line *et al*., 1998 Morishita *et al*., 1997 Schoeni & Wong, 1994 Svetoch & Stern, 2010
	Competitive exclusion of human pathogenic *C. jejuni* strains by characterized hyper-colonizing *C. jejuni* strains	Needs further research regarding applicability under commercial settings	Chen & Stern, 2001 Calderon-Gomez *et al*., 2009

Prebiotics	Reduction in cecal *Campylobacter* load in animals fed mannanoligosaccharide or xylanase	Limited reducing potential	Baurhoo *et al*., 2009; Fernandez *et al*., 2000
Antibiotic dissection	A subtype I of *Megamonas hypermegale* specific for a *C. jejuni*-suppressive treatment	*In vivo* experiments are needed to prove *C. jejuni* exclusion in poultry	Scupham *et al*., 2010

Table 2 On-farm intervention measures to reduce the cecal *Campylobacter* load of already-colonized broiler chickens

Intervention measure	Effect on cecal *Campylobacter* numbers	Points of attention	References
Drinking water treatment	Reduced *C. jejuni* count on cloacal swabs of broilers receiving monocaprin	Correlation between cecal *C. jejuni* load and numbers on cloacal swabs?	Hilmarsson *et al*. (2006)
	Reduced cecal *Campylobacter* counts from broilers receiving caprylic acid	Inconsistent results	Metcalf *et al*. (2011)
	Reduced crop and pre-chill carcass contamination		Byrd *et al.*, 2001
Feed additives	Reduced cecal *Campylobacter* load in broilers fed organic acids	Further data needed to prove consistency	Hilmarsson *et al*. (2006) Solis de los Santos *et al*. (2010)
Bacteriophages	Reduced cecal *Campylobacter* load by 2 log when phages are administered through the feed	Long-term efficacy? Genomic instability?	Wagenaar *et al*. (2005) El-Shibiny *et al*. (2009) Carvalho *et al*. (2010)
Passive immunization	Minor reduction (80%-95%) in fecal bacterial counts upon treatment with Ig preparations	Optimization and experimental trials in industrial or farm settings are needed to prove efficacy	Tsubokura *et al*. (1997)
Bacteriocins	Supplementation of BCN 760 in drinking water capable of complete eradicating *C. jejuni* in 90% of the cases	Safety? Long-term efficacy?	Svetoch & Stern (2010)

1.3.1 Hygienic and biosecurity farming practices

Good hygienic farming practices constitute a strategy aimed to prevent the introduction of *Campylobacter* into a flock by a combination of hygiene and biosecurity measures. A Belgian quantitative microbial risk assessment showed that the incidence of human campylobacteriosis in Belgium would be reduced by 32%, 53% and 77% when the *Campylobacter* flock prevalence is reduced by 25%, 50% or 75% respectively (Messens *et al*., 2007). Application of specific hygienic measures during the rearing period, such as washing hands before entering the chicken house, the use of separate boots for each broiler house, footbath disinfection when entering a broiler house and a high standard of cleaning and disinfection of the drinking water equipment may significantly reduce the risk of *Campylobacter*-colonized broiler flocks (van de Giessen *et al*., 1996; Evans & Sayers, 2000). After introduction of hygienic and biosecurity measures, including the control of rodents and insects, in two Dutch broiler farms, the ratio of *Campylobacter*-colonized flocks compared to the total amount af flocks decreased from 12/18 at one farm and 4/4 at the second farm to 2/9 and 5/12, respectively (van de Giessen *et al*., 1998). In the UK, the implementation of an intervention trial, based on a standard hygiene protocol for personnel and proper disinfection of the broiler house prior to stocking, reduced the prevalence of *Campylobacter* infection in the broiler population from 80% to < 40% (Gibbens *et al*., 2001). It has been demonstrated that the prevalence of broiler flocks colonized with *Campylobacter* can be reduced from 51.4% to 15.4% by placing fly screens in broiler houses (Hald *et al*., 2007). In Denmark, biosecurity strategies to control *Campylobacter* on-farm were intensified in 2003 leading to a decrease of *Campylobacter*-colonized flocks from 43% in 2002 to 27% in 2007 (Rosenquist *et al*., 2009). These findings suggest that proper application of biosecurity measures can lead to reduced *Campylobacter* colonization in poultry. However, because broiler chickens are under a constant contamination pressure, biosecurity measures alone will not be sufficient to solve the problem.

1.3.2 Drinking water treatment

By treating the drinking water for poultry flocks, the risk of the animals to become infected might be reduced, probably through a reduction in bacterial numbers both in the drinking water and the crop. In this way, *Campylobacter* is less likely to reach the ceca and transmission throughout the flock might be reduced or prevented.

In vitro studies have demonstrated that organic acids have a strong bactericidal effect on *Campylobacter* spp. and addition of these acids to the drinking water on poultry farms could prevent transmission through broiler flocks (Chaveerach *et al*., 2002; Chaveerach *et al*., 2004b). Preventive addition of monocaprin, the mono-acylglycerol of capric acid (Thormar *et al*., 2006), to the drinking water resulted in a reduced *C. jejuni* count on cloacal swabs of approximately 2 logs, two days after experimentally inoculated chicks were co-housed with *C. jejuni*-free birds (Hilmarsson *et al*., 2006). Extending the treatment period, however, resulted in a reduced effectivity of the treatment at days 5 and 10 after birds were co-housed. Moreover, on day 13 no significant reduction was observed in cecal *C. jejuni* counts of birds receiving monocaprin, indicating that this treatment did not prevent *C. jejuni* spread from artificially infected to non-infected birds. Chlorinating the drinking water is helpful as it reduces the risk for *Campylobacter* colonization (Ellis-Iversen *et al*., 2009). Chlorination of flock drinking water (with 2-5 ppm chlorine) under commercial production practices in the US in 2002 did, however, not result in a reduced *Campylobacter* prevalence in the birds receiving treated water (Stern *et al*., 2002).

Alternatively, addition of monocaprin to drinking water from the last three days before slaughter resulted in a reduced *C. jejuni* count of approximately log 1.85 on cloacal swabs of naturally infected birds, compared to control birds (Hilmarsson *et al*., 2006). Addition of 0.44% (vol/vol) lactic acid in the drinking water during pre-slaughter feed withdrawal reduced the incidence of both crop (by 22.8%) and pre-chill carcasses (by 14.7%) contamination with *Campylobacter* compared to control groups (Byrd *et al.*, 2001). Finally, Metcalf *et al*. (2011) observed a 3 log reduction in cecal *Campylobacter* counts of 14-d-old chicks receiving drinking water supplemented with 0.175% (wt/vol) caprylic acid. However, this decrease was not consistent and moreover absent when applying different caprylic acid doses.

1.3.3 Feed additives

Changes in the composition of the feed can promote gastro-intestinal health and thus contribute to the control of *Campylobacter* in poultry. Plant-derived antimicrobial feed additives can be administered from day-of-hatch to prevent broiler chickens to become colonized and to reduce *Campylobacter* transmission throughout the flock. Also in this application, the observed effect is largely due to the anti-*Campylobacter* effect in the crop of the animals.

Next to their application in drinking water, organic acids might also be used as feed additives to reduce *Campylobacter* prevalence in poultry. However, *in vivo* trials demonstrated only a limited effect of feed acidification on *C. jejuni* prevalence in broiler flocks. At most it could delay the onset of colonization (Heres *et al.*, 2004; Line & Bailey, 2006). Broilers that were fed fermented liquid feed, i.e. a moistened feed with a high number of lactobacilli, a high concentration of lactic/acetic acid and a pH of 4, were less likely to shed *Campylobacter* after oral infection (Heres *et al*., 2003). However, at the end of the trial no significantly different *C. jejuni* counts in the ceca could be observed compared to chickens on a standard feed. The higher level of lactic acid in combination with a low pH in the crop was suggested to reduce the probability for *Campylobacter* to reach the ceca. In a later experiment, individually housed chickens that were fed acidified feed were found to be less susceptible to *Campylobacter* infection compared to control birds, as less chickens became colonized at equal inoculation doses (Heres *et al.*, 2004). Also caprylic acid leads to reduced colonization in 10-day-old chicks when given preventively (Solis de los Santos *et al*., 2008). In contrast, addition of butyrate to the feed was not able to reduce cecal *Campylobacter* colonization in a seeder model using two-week-old broilers (Van Deun *et al*., 2008a). Skanseng *et al*. (2010) found little effect when supplementing only formic acid to the feed, but a combination of 2% formic acid with 0.1% sorbate prevented *C. jejuni* colonization in chicks. Finally, it was demonstrated that the addition of a medium-chain fatty acid mixture to the feed at 1% reduces the probability of broilers becoming colonized (van Gerwe *et al*., 2010). Administration of large molecules that interfere with *Campylobacter* adhesion to the host cell is successful *in vitro* but suffers from premature metabolic breakdown in the broiler chicken GI tract (Wittschier *et al*., 2007). Finally, cecal colonization of birds receiving plant-protein-based feed was significantly lower compared to birds receiving animal-protein-based feed or a combination of plant- and animal-protein sources (Udayamputhoor *et al*., 2003).

Alternatively, colonized broiler chickens might be fed pulse doses of the additives for a certain period, just before slaughter, aiming at reducing the cecal *Campylobacter* load and reducing carcass contamination after slaughter. In this application one aims to reduce the *Campylobacter* numbers in the ceca of already colonized birds. To reach the cecum efficiently, additives are often coated on/encapsulated in carrier material that will prevent premature degradation along the GI tract and assure efficient release of the active compound into the gut (Van Immerseel *et al*., 2004). Addition of monocaprin to the feed from the last three days before slaughter, resulted in a reduced *C. jejuni* count on cloacal swabs of naturally infected birds (Hilmarsson *et al*., 2006). In addition, Solis de los Santos *et al*. (2010) observed

a considerable reduction (of several logs) in cecal *Campylobacter* numbers when caprylic acid was given from three days before slaughter, in already colonized market-aged broilers. This reduction was strikingly not accompanied by an altered cecal microbial population.
As the available *in vivo* results are limited and moreover contradictory, it cannot be univocally be determined what the contribution of feed additives will be to control cecal *Campylobacter* colonization. Preventive supplementation from day-of-hatch, rather than aiming for reduced cecal *Campylobacter* numbers in already colonized birds, seems most promising.

1.3.4 Bacteriophage application

Bacteriophage application to reduce cecal *Campylobacter* colonization in poultry is promising (Carrillo *et al*., 2005; Wagenaar *et al*., 2005). Results indicate an immediate drop in *C. jejuni* numbers of approximately three logs in already-colonized chicken ceca (Wagenaar *et al*., 2005). After five days, however, bacterial counts stabilized at a level one log lower compared to control birds, an effect also observed when phages were given prophylactically. Also El-Shibiny *et al*. (2009) observed an immediate (after two days) two-log cfu/g reduction in cecal *C. jejuni* and *C. coli* levels. Despite the fact that *Campylobacter*, after a sudden drop, seems to re-establish itself to nearly its original counts, results do indicate that bacteriophages can possibly be successfully applied in broilers to reduce the cecal bacterial load just before slaughter. Further research in this area showed that administering phages in the feed is more efficient than oral gavage (Carvalho *et al*., 2010). This study revealed an initial drop, already after two days, of approximately two logs in the numbers of *C. jejuni* in the fecal material of infected one-week-old birds using a phage cocktail. Moreover, *C. jejuni* did not regain its original counts throughout the experimental period, which was ended seven days after phage administration had started.
Although the use of phage products in broilers seems to be a promising way to reduce cecal colonization with *C. jejuni*, questions regarding both immediate and long-term efficacy, consumer safety and application methods arise (Hagens & Loessner, 2010). Safety concerns should not be a main obstacle as phages are highly specific and can only infect a limited range of host bacteria. Moreover, their oral consumption, even at very high levels, is believed to be completely harmless to humans. Answers concerning the efficacy seem to be more complex, especially if long-term efficacy of the phage product has to be ensured. In the study of El-Shibiny *et al*. (2009) it was shown that 2% of the *Campylobacter* population exposed to virulent phages in the chicken, developed phage-resistance. These resistant types remained a

minor component of the population. Carvalho *et al*. (2010) isolated phage-resistant *Campylobacter* strains from phage cocktail-administered chicks at a frequency of 13%. Strikingly, also before phages were applied resistance was observed, although at a lower frequency (6%). Nevertheless, an increase in the resistant *Campylobacter* population was observed after applying phages, suggesting that phages might have selected for resistant strains. Because further information on this topic is lacking, long-term efficacy of phages to control *C. jejuni* in poultry cannot be ensured.

1.3.5 Vaccination

Studies showed that if antibodies against *C. jejuni* are present in the chick, be it due to transfer of maternal antibodies to, or through immunization, the ability of *C. jejuni* to colonize these chicks is dramatically reduced (Sahin *et al*., 2003; Shoaf-Sweeney *et al*., 2008). This indicates that it may be possible to reduce *Campylobacter* colonization in broilers by immunization-based approaches (Shoaf-Sweeney *et al*., 2008). Several vaccination studies aiming to reduce the susceptibility of broiler chickens for *Campylobacter* colonization have been reported, although with variable results.

In ovo vaccination by injection of heat-killed *C. jejuni* in the amniotic fluid resulted in an increase in IgA antibodies (Noor *et al*., 1995). However, the consequences on a subsequent challenge were not studied. Intraperitoneal immunizations of chickens with killed *C. jejuni* whole cells at 16 and 29 days of age reduced the intestinal colonization, which was associated with an increase in specific IgY in intestinal secretions (Widders *et al*., 1996). In addition, Rice *et al*. (1997) demonstrated some reduction of *Campylobacter* colonization of chicks orally vaccinated with formalin-killed *C. jejuni* whole cells in combination with *Escherichia coli* heat-labile toxin when compared to non-vaccinated control birds.

For subunit vaccines, flagellin and OMPs have been tested and are considered useful candidates. In a study involving immunization of chickens with heat-killed *C. jejuni*, intestinal colonization upon challenge was reduced, with flagellin and a 67 kDa protein showing up as the immunodominant antigens (Widders *et al*., 1998). Vaccination of chickens with a hybrid protein, containing part of the *C. jejuni* FlaA and the B-subunit of *E. coli* heat-labile toxin, elicited specific antibodies against *C. jejuni* flagellin and reduced colonization of the chickens after challenge (Khoury & Meinersmann, 1995). Chickens orally immunized with an avirulent recombinant *Salmonella* strain carrying the *Campylobacter cjaA* gene, encoding a highly immunogenic lipoprotein which is conserved among different

Campylobacter serotypes, developed serum IgY and mucosal IgA antibody responses against *Campylobacter* and *Salmonella* OMPs and were protected against cecal colonization with a heterologous wildtype *C. jejuni* strain (Wyszynska *et al*., 2004). A more recent study evaluated the potential use of a heterologous vaccine for *Campylobacter* control in poultry using substantially more animals (Buckley *et al*., 2010). Upon vaccination with a *Salmonella* Typhimurium *ΔaroA* mutant, expressing CjaA as a plasmid-encoded fusion to tetanus toxin, birds had significantly reduced cecal *C. jejuni* counts of approximately $\log_{10}$ 1.4 cfu/g three and four weeks after *C. jejuni* inoculation, compared to unvaccinated control birds. This protection was associated with increased levels of CjaA-specific serum IgY and biliary IgA in the vaccinated chicks. Also in this study, a group of chicks receiving a vaccine strain containing the non-recombinant plasmid was incorporated. These animals were not protected, indicating that the protective effect observed in the birds receiving the heterologous vaccine, expressing CjaA, is due to responses directed against CjaA rather than competitive or cross-protective effects mediated by the carrier. Broiler chicks orally gavaged with live *Salmonella*-vectors expressing *Campylobacter* Omp18/CjaD, CjaA and ACE393 at day-of-hatch and inoculated with *C. jejuni* at 21 days of age, had higher serum IgG and mucosal sIgA levels, as well as reduced ileal *C. jejuni* counts at day 32 compared with control birds (Layton *et al*., 2010). Vaccination with the Omp18/CjaD peptide-expressed vector was most effective and *Campylobacter* could not be recovered from ileal samples. However, the cecal *Campylobacter* load, a better indicator for the colonization level in broilers (Beery *et al*., 1988), was not determined.

Zeng *et al*. (2009) showed that specific CfrA antibodies can block the function of this protein, diminishing ferric enterobactin-mediated growth promotion under iron-restricted conditions in a dose-dependent way. As inactivation of the *cfrA* gene completely eliminates *Campylobacter* colonization in chicks and CfrA is both expressed and immunogenic in chickens experimentally infected with *C. jejuni*, CfrA could be a promising candidate for a subunit vaccine for *Campylobacter* control in poultry (Zeng *et al*., 2009), but this hypothesis has yet to be tested.

Despite all this research, an effective vaccine to combat cecal *Campylobacter* colonization in poultry is not yet available. Recently, however, several critical colonization determinants have been identified which may be good targets for the development of effective vaccines to eradicate this zoonotic pathogen from poultry flocks. These potential vaccine candidates must be expressed during colonization in chicks and should ideally be highly immunogenic, conserved and prevalent among *C. jejuni* isolates. Bacterial OMPs are regarded as promising

vaccine components because of their accessibility for the host immune system and the key roles they play in the host-bacterium cross-talk (Zeng *et al*., 2009). CadF and CfrA may therefore hold much promise for such applications. Not only are they highly conserved and prevalent in *C. jejuni* strains, but these surface-exposed proteins are also highly immunogenic in chicks (Shoaf-Sweeney *et al*., 2008; Zeng *et al*., 2009). *C. jejuni* periplasmic PEB1 can possibly be transported across the outer-membrane and is highly immunogenic in humans. Whether this immunogenicity can be extended to the chicken host is not known. Finally, the secreted CiaB and transmembrane Tlp10 may be immunogenic but their precise role during chick colonization has yet to be determined. Also *C. jejuni* surface-exposed polysaccharide structures may be promising candidates for subunit vaccines. Indeed, several genes essential for successful colonization (*kpsM*, *flaA*, *flgK*, *pglH*, *maf5* and *cj1324*) are involved in SACS biosynthesis. However, most SACS are highly variable and implicated in immune evasion in humans. Whether this can be extended to the chicken host is not clear. In any case, identification of conserved polysaccharide epitopes of SACS is critical for exploiting these structures for vaccine application. Finally, a plethora of other *C. jejuni* factors are indispensable for chicken gut colonization (see 1.2.2). Gene products of most of them are, however, not known to be surface-expressed, but rather reside in the peri- or cytoplasm where they exert their vital roles. As a consequence, they do not come in direct contact with the chick immune system and consequently are expected to have limited efficacy in vaccine applications.

1.3.6 Passive immunization

Experimental studies have shown that chick colonization can be inhibited by using antibodies. *Campylobacter*-specific maternal antibodies protect young chickens from colonization (Sahin *et al*., 2003). Pre-incubation of *Campylobacter* with rabbit hyper-immune antiserum or chicken bile antibodies increased the dose required to colonize the chicken cecum (Stern *et al*., 1990b). Oral administration of bovine or chicken Ig preparations from respectively milk or eggs of hyper-immunized animals, conferred a marked protection against challenge with *C. jejuni* in chickens (Tsubokura *et al*., 1997). Fecal bacterial counts were reduced by >99% (prophylaxis) or 80% - 95% (post-colonization) using an antibody preparation. The mean number of bacteria quickly increased, however, after ending the colonization-reducing addition with antibodies. This strategy might thus be applied to reduce cecal numbers of bacteria immediately before slaughter.

1.3.7 Competitive exclusion, probiotics and prebiotics

Although the exact exclusion mechanism is not fully understood, experiments have shown that competitive exclusion microflora can prevent *Campylobacter* colonization of the chicken gut. Competitive exclusion is a prophylactic measure that aims at increasing the resistance of chicks to *Campylobacter* infection.

Undefined bacterial mixtures have been demonstrated to effectively control *Campylobacter* infections in young chicks artificially challenged with a chicken *C. jejuni* isolate (Soerjadi *et al*., 1982; Soerjadi-Liem *et al*., 1984). In another study, however, this protective effect was not observed (Stern *et al*., 1988). The efficacy of competitive exclusion depends on cultivation methods and storage of the microbiota. It was found that the efficacy of using competitive exclusion microflora decreased with storage of the cultures (Stern, 1994). Different culture preparation techniques, with respect to the level of anaerobic culture, degree of epithelial scraping of the ceca, media used for subculturing and incubation temperature, resulted in different degrees of protection against colonization with *Campylobacter* spp. (Stern *et al*., 2001a). However, Schoeni & Wong (1994) concluded that protection by aerobically grown cultures was not statistically different from that obtained with anaerobically grown cultures.

Later, attempts have been made to develop defined microbiota. A standard feed supplemented with the yeast *Saccharomyces boulardii* did not significantly affect cecal *Campylobacter* colonization of experimentally challenged chickens (Line *et al*., 1998). The use of a probiotic containing *Lactobacillus acidophilus* and *Enterococcus faecium* in chicks, during the first three days of rearing, reduced both *C. jejuni* fecal shedding and jejunal colonization in colonized market-aged broilers, experimentally infected with *C. jejuni* six hours after the first oral administration of the probiotic, with 70% and 27% respectively (Morishita *et al*., 1997). Administration of competitive exclusion cultures of *Citrobacter diversus*, *Klebsiella pneumoniae* and *E. coli* effectively prevented or reduced *C. jejuni* colonization in chickens after *Campylobacter* inoculation (Schoeni & Wong, 1994). This protection was enhanced by feeding mannose to the chickens. In a simulated chicken digestive tract model, addition of *L. acidophilus*, *L. fermentum*, *L. crispatus* and *L. brevis* exerted an antagonistic effect on *C. jejuni* (Chang & Chen, 2000). A *Lactobacillus* strain was isolated from an adult chicken gut that showed bactericidal effects against *Campylobacter in vitro*, probably by the production of organic acids and an anti-*Campylobacter* peptide (Chaveerach *et al*., 2004a). Two promising antagonistic isolates (*L. salivarius* NRRL B-30514 and *Paenibacillus polymyxa* NRRL-B-

30509), acting as probiotics, were ineffective to control *Campylobacter*, whether the isolates were fed to chicks before or after artificial challenge with *C. jejuni* (Stern *et al*., 2008). These isolates were, however, able to produce bacteriocins which are able to reduce the *Campylobacter* load in the gut of colonized birds (see further).

It has been demonstrated that it is possible to use combinations of (heterologous) *C. jejuni* chicken isolates for the competitive exclusion of human pathogenic *C. jejuni* strains in poultry (Chen & Stern, 2001). Circulation of uncharacterized environmental *Campylobacter* strains in commercial poultry flocks could possibly be biologically controlled by a characterized hyper-colonizing *C. jejuni* strain. Australian researchers identified such a strain that was capable of displacing other colonizing strains and maintain itself in the chicken GI tract for the entire 56-day broiler production cycle, without being displaced by other (hyper-)colonizing strains, once colonization was established (Calderon-Gomez *et al*., 2009).

With an approach called antibiotic dissection, day-old turkey poults were inoculated with cecal contents of *Campylobacter*-free adult turkeys after which the microbial communities in these poults were modified by different antibiotic treatments. It was investigated which modified intestinal microbiota was able to outcompete a *Campylobacter* challenge. Molecular examination of the constituents of these communities detected a subtype I of *Megamonas hypermegale* to be specific for a *C. jejuni*-suppressive application (Scupham *et al*., 2010). *In vivo* competition experiments with *M. hypermegale* isolates of both subtypes will be necessary to prove *C. jejuni* exclusion in poultry.

Finally, addition of mannanoligosaccharide to the feed of naturally infected birds and xylanase to the feed of artificially infected broilers, as prebiotics, resulted both in a minor, although significant decrease in cecal *C. jejuni* counts in these animals (Fernandez *et al*., 2000; Baurhoo *et al*., 2009).

1.3.8 Bacteriocin application

Svetoch & Stern (2010) recently reviewed bacteriocin application to reduce cecal *Campylobacter* counts in broiler chickens of colonized flocks. Applying purified encapsulated bacteriocin from either *L. salivarius* NRRL B-30514 or *P. polymyxa* NRRL-B-30509 to the feed during three days before euthanization led to a reduction of cecal *Campylobacter* colonization in broiler chickens, orally gavaged with *C. jejuni* at day-of-hatch, by at least six logs. However, birds were only seven to ten days of age and birds at slaughter age have not been examined in this study. Further research by these authors led to the identification of two

more bacteriocin-producing isolates with marked anti-*Campylobacter* activity: *E. durans/faecium/hirae* (NRRL B-30745), producing bacteriocin BCN E 760 and *E. faecium* (NRRL B-30746), producing BCN E 50-52. Both bacteriocins were able to tremendously lower (> 6 $\log_{10}$ cfu/g or below detectable levels) the cecal *C. jejuni* load in inoculated broilers. Also in naturally-colonized market-aged broilers these bacteriocins were effective. BCN E 760 reduced the cecal *Campylobacter* load in these animals from an average of $\log_{10}$ 6.2 cfu/g to undetectable levels when added to the feed four days before slaughter. BCN E 50-52 at 10.8 mg per bird was able to reduce cecal colonization by > 5 $\log_{10}$ cfu/g when added to the drinking water three days before slaughter. Supplementing BCN 760 in drinking water at 3.5 to 25 mg per bird for three days before slaughter was most effective, resulting in a complete elimination of *C. jejuni* in 90% of the cases or else, a reduction of over six logs. The safety of these bacteriocins was confirmed by conducting experiments on monkey and human cell cultures as well as in treated mice and chickens. Santini *et al*. (2010) very recently reported both marked *in vitro* and *in vivo* activity for *Bifidobacterium longum* PCB 133 toward *Campylobacter*. After two weeks of daily administration, excreted *B. longum* PCB 133 counts were still high in the feces of orally gavaged chicks, even after a wash-out period of six days and *C. jejuni* numbers were significantly reduced by one log after this administration period.

1.4 GENERAL CONCLUSION

Campylobacter jejuni is the most common cause of bacterial-mediated diarrheal disease in humans worldwide and poses a serious health burden in industrialized countries. Despite their fragile nature, *Campylobacter* spp. are highly adapted to the broiler chicken gut. In contrast to the severe inflammatory disease that is often observed in humans, *C. jejuni* colonizes chickens asymptomatically and colonized birds carry a very high bacterial load in their gastro-intestinal (GI) tract, especially the ceca. *C. jejuni* is typically detected in broiler flocks only starting from two to four weeks of age, which coincides with a reduction in maternally-derived anti-*C. jejuni* antibodies in these chicks. After this lag-phase, birds become more susceptible and colonization is detected in most flocks. Rapid transmission through the fecal-oral route results in colonization of all birds in a flock only a few days after the first bird is colonized. The inefficient immune response that is induced during commensal colonization with *C. jejuni* is not able to clear this zoonotic pathogen from the chicken gut. As a consequence, animals remain colonized up to slaughter which results in an enormous amount of contaminated

carcasses after processing. Increasing evidence suggests that *Campylobacter*-contaminated poultry meat is the most important source of campylobacteriosis in humans. While hygienic measures at the farm and control measures during carcass processing can have some effect on the reduction of *Campylobacter* numbers on the retail product, intervention at the farm-level by reducing colonization of the ceca should be definitely taken into account in the overall control policy. However, at present no effective strategy exists that consistently reduces *Campylobacter* counts in the ceca of broiler chicks. As a consequence, the prevalence of *Campylobacter* in broiler flocks and on broiler carcasses, and thus the incidence of campylobacteriosis in humans remain strikingly high.

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CHAPTER 2: AIMS

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Campylobacter jejuni is the most common cause of bacterial-mediated diarrheal disease in humans worldwide and poses a serious health burden in industrialized countries. Poultry is considered the most important source for campylobacteriosis in humans. *C. jejuni* is highly adapted to the chicken gut and colonized birds carry a very high bacterial load, especially in their ceca. Slaughtering infected broiler flocks results in an enormous amount of contaminated carcasses after processing which can transmit the pathogen to humans. Since the cecum is the only amplification point of *C. jejuni* throughout the entire food chain, on-farm intervention measures that reduce colonization of the ceca may have a great impact on the number of campylobacteriosis cases in humans. The use of antimicrobial agents for this purpose is not allowed and at present no alternative strategy exists that consistently reduces *C. jejuni* in broiler flocks.

Therefore, the general scientific aim of this project was to develop an intervention measure that consistently reduces *C. jejuni* numbers in the ceca of broiler chickens by at least a thousand-fold, which would lead to a reduction in the number of human campylobacteriosis cases by a ten-fold (Rosenquist *et al*., 2003, Messens *et al*., 2007; Reich *et al*., 2008).

For this purpose,

- organic acids (chapter 3.1) and plant-derived antimicrobial compounds (chapter 3.2) were screened and tested for their anti-*C. jejuni* activity using several *in vitro* and *in vivo* models mimicking the *C. jejuni*-chicken host interaction;
- the potential contribution of passive immunization for *C. jejuni* control in broilers was investigated (chapter 3.3).

CHAPTER 3: EXPERIMENTAL STUDIES

3.1 EFFICACY OF WATER AND IN-FEED APPLICATIONS OF MEDIUM-CHAIN FATTY ACIDS ON *CAMPYLOBACTER JEJUNI* COLONIZATION AND TRANSMISSION IN BROILER CHICKENS

3.1.1 Intestinal mucus protects *Campylobacter jejuni* in the ceca of colonized broiler chickens against the bactericidal effects of medium-chain fatty acids

3.1.2 Application of medium-chain fatty acids in drinking water increases the *Campylobacter jejuni* colonization treshold in broiler chicks

3.2 THE CINNAMON-OIL INGREDIENT *TRANS*-CINNAMALDEHYDE FAILS TO TARGET *CAMPYLOBACTER JEJUNI* IN THE BROILER CHICKEN CECUM DESPITE MARKED ACTIVITY *IN VITRO*

3.3 PASSIVE IMMUNIZATION TO PREVENT AND REDUCE *CAMPYLOBACTER JEJUNI* COLONIZATION AND TRANSMISSION IN POULTRY

CHAPTER 3: EXPERIMENTAL STUDIES

3.1 EFFICACY OF WATER AND IN-FEED APPLICATIONS OF MEDIUM-CHAIN FATTY ACIDS ON *CAMPYLOBACTER JEJUNI* COLONIZATION AND TRANSMISSION IN BROILER CHICKENS

3.1.1 **Intestinal mucus protects *Campylobacter jejuni* in the ceca of colonized broiler chickens against the bactericidal effects of medium-chain fatty acids**

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Poultry Science (2010), 89:1144-1155

SUMMARY

Campylobacter jejuni is the most common cause of bacterial-mediated diarrheal disease worldwide. As poultry and poultry products are a major source of *C. jejuni* infections in humans, efforts should be taken to develop strategies to decrease *Campylobacter* shedding during primary production. For this purpose, the efficacy of medium-chain fatty acids (MCFA) as feed additives to control *C. jejuni* colonization in broiler chickens was analyzed. First, the antimicrobial activity of the MCFA caproic, caprylic, and capric acid on *C. jejuni* was evaluated *in vitro*. Minimal inhibitory concentrations (MIC) were 0.25 m*M* for caproic and 0.5 m*M* for caprylic and capric acid at pH 6.0, and 4 m*M* for all 3 compounds at pH 7.5. Time-kill curves revealed strong bactericidal properties of the tested compounds toward *C. jejuni* at pH 6.0. Concentrations as low as 4 m*M* of caprylic and capric acid, and 16 m*M* of caproic acid killed all bacteria within 24 h. Capric acid had the highest activity, with concentrations of 4 m*M* killing all bacteria within the hour. Together these data show a profound bactericidal, dose-dependent activity of the tested MCFA toward *C. jejuni in vitro*. For this reason, the effect of these 3 MCFA on *C. jejuni* was evaluated *in vivo*. The addition of any of the acids to the feed, from 3 day before euthanization, was not capable of reducing cecal *Campylobacter* colonization in 27-day-old broilers, experimentally infected with *C. jejuni* at 15 days of age. Using a 'cecal loop' model, sodium caprate was not able to reduce cecal *Campylobacter* counts. When time-kill curves were conducted in the presence of chick intestinal mucus, capric acid was less active against *C. jejuni*. At 4 m*M*, all bacteria were killed only after 24 h. Thus, despite the marked bactericidal effect of MCFA *in vitro*, supplementing these acids to the feed does not reduce cecal *Campylobacter* colonization in broiler chickens under the applied test conditions, probably due to the protective effect of the mucus layer.

Key words: medium-chain fatty acid; *Campylobacter jejuni*; broiler chicken; chicken intestinal mucus

INTRODUCTION

Today, *Campylobacter jejuni* is the most common cause of bacterial-mediated diarrheal disease in humans, worldwide (Blaser, 1997; Zilbauer *et al*., 2008). Most *Campylobacter* infections are self-limiting, seldom requiring antimicrobial therapy. In a small number of cases however, an infection may lead to long-term complications of which Guillain-Barré syndrome, an acute autoimmune disease affecting the peripheral nervous system with an incidence of one in 1000 infected people, is the most serious (Butzler, 2004). Poultry and poultry products are considered one of the most significant sources for *Campylobacter* infections (Vellinga & Van Loock, 2002; Friedman *et al*., 2004; Wingstrand *et al*., 2006). It is suggested that chickens as a reservoir account for 50% to 80% of human campylobacteriosis cases (EFSA, 2010). Many efforts aimed at minimizing contamination of poultry with *Campylobacter* failed however to reduce the number of reported poultry meat consumption-related human campylobacteriosis cases (EFSA, 2009).

A significant correlation exists between *Campylobacter* concentrations in the intestinal contents and on chicken carcasses after processing (Herman *et al*., 2003; Rosenquist *et al*., 2003). Even small amounts of cecal contents (5 mg) can remarkably increase the numbers of *Campylobacter* on eviscerated broiler carcasses (Berrang *et al*., 2004) as *Campylobacter* are found abundantly in the large intestine, cloaca and ceca, where 10^5-10^9 cfu/g fecal content have been observed (Corry & Atabay, 2001). A recent study stated that the number of campylobacteriosis cases in Belgium associated with consumption of chicken meals could be reduced by 84% by lowering *Campylobacter* numbers on chicken carcasses by 2 log (Messens *et al*., 2007), while a Danish quantitative risk assessment showed that this reduced contamination level would decrease the number of campylobacteriosis cases 30 times (i.e. a 97% reduction) (Rosenquist *et al*., 2003). This indicates that measures taken to reduce *Campylobacter* colonization in the intestinal tract of poultry during primary production have potential for reducing contamination of poultry products, and thus the incidence of human campylobacteriosis. For this purpose, a plethora of different approaches has been examined over the past few years, all with varying degrees of success. Amongst others, vaccination (Noor *et al*., 1995; Widders *et al*., 1996; *Rice et al*., 1997; Wyszynska *et al*., 2004) and passive immunization (Sahin *et al*., 2003) of chickens has been studied. Also, the effect of feeding broilers competitive exclusion microbiota (Soerjadi-Liem *et al*., 1984; Schoeni & Wong, 1994; Stern *et al*., 2001), bacteriophages (Wagenaar *et al*., 2005), bacteriocins (Line *et al*., 2008), organic acids (Byrd *et al*., 2001; Chaveerach *et al*., 2004 ; Van Deun *et al*., 2008a)

or their derivatives (Hilmarsson *et al*., 2006), and antibiotics (Farnell *et al*., 2005) have been assessed. Nonetheless, to date there is no effective, reliable, and practical intervention measure available to reduce colonization of the broiler gut with *Campylobacter* (Lin, 2009).

Antibacterial growth promoting agents used in the rearing of broiler chickens have been banned by the European Union due to concerns regarding the use of chemical additives in food production (Dibner & Richards, 2005). Also for therapeutic purposes, the use of antibacterial agents should not be encouraged. Indeed, there is increasing evidence of the development of antimicrobial resistant *Campylobacter* strains which may compromise the treatment of human campylobacteriosis (Smith *et al*., 1999; Engberg *et al*., 2001; Rao *et al*., 2005; Gibreel & Taylor, 2006; Moore *et al*., 2006; Luangtongkum *et al*., 2009). Using fatty acids instead could be a promising alternative because of their antimicrobial activities against a wide range of microorganisms (Kabara *et al*., 1972; Bergsson *et al*., 2002; Van Immerseel *et al*., 2004a; Boyen *et al*., 2008), including *Campylobacter* (Thormar *et al*., 2006; Houf *et al*., 2007). Van Immerseel *et al*. (2006) stated that of all fatty acids, medium-chain fatty acids (MCFA) have probably the greatest antimicrobial activity against *Salmonella*, which like *Campylobacter* is a Gram-negative bacterium causing gastro-enteritis. Recently, it was shown that the supplementation of the MCFA caprylic acid to broiler feed could reduce *C. jejuni* colonization in these animals, both in a prophylactic way (Solis de los Santos *et al*., 2008a) as well as therapeutically (Solis de los Santos *et al*., 2008b; Solis de los Santos *et al*., 2009).

The aim of this study was to investigate the applicability of MCFA to obtain a 3 log reduction in cecal *Campylobacter* levels in infected chickens before slaughter, thereby aiming at lowering carcass contamination and eventually the number of human campylobacteriosis cases. First, a thorough *in vitro* evaluation of the antimicrobial activity of MCFA on *C. jejuni* was performed. Secondly, the *in vivo* effect of these acids toward *C. jejuni* was analyzed.

MATERIALS AND METHODS

Experimental animals

Day-of-hatch Ross broiler chickens of both sexes from a local farm were raised in group until treatment and provided with a commercial starter feed and water ad libitum. Husbandry, euthanasia methods, experimental procedures and bio-safety precautions were approved by the Ethical Committee (EC) of the Faculty of Veterinary Medicine, Ghent University, Ghent,

Belgium (EC numbers: 2009/31, 2009/56 and 2009/114wi). Chicks were examined for the presence of *Campylobacter* in mixed fecal samples and proved to be *Campylobacter* free.

Bacterial strains and culture conditions

Campylobacter jejuni strains KC 40 and KC 96.1 from poultry origin as well as R-27451, R-27456, R-27467 and R-27473 (kindly provided by Prof. Dr. Peter Vandamme, Ghent University, Ghent, Belgium) isolated from human patients suffering from campylobacteriosis, were used to perform MIC assays. These strains were chosen based on their difference in invasion efficiency in primary cecal epithelial cells from chickens, as determined by Van Deun *et al*. (2008b). For all further experiments, *C. jejuni* strain KC 40 was used. This strain colonizes chickens to a high level (Van Deun *et al*., 2008b). Bacteria were routinely cultured in Nutrient Broth No.2 (NB2, CM0067; Oxoid Ltd., Basingstoke, Hampshire, UK) supplemented with Modified Preston *Campylobacter*-selective supplement (SR0204E; Oxoid) and *Campylobacter*-specific growth supplement (SR0232E; Oxoid), at 42°C under microaerobic conditions (5% O_2, 5% CO_2, 5% H_2, 85% N_2). *C. jejuni* bacteria were enumerated by preparing 10-fold dilutions in Hank's Balanced Salt Solution (HBSS; GIBCO-BRL, Invitrogen, Carlsbad, CA) and plating on modified charcoal cefoperazone deoxycholate agar (mCCDA, CM0739; Oxoid) supplemented with CCDA selective supplement (SR0155E; Oxoid) and *Campylobacter*-specific growth supplement (SR0232E; Oxoid), followed by microaerobic incubation at 42°C for 22 h.

Collection of chicken intestinal mucus

Commercial 28-week old brown laying hens were euthanized and the small intestine was collected and gently rinsed with PBS to remove fecal material. The mucus was scraped from the mucosa with a glass slide covered in parafilm, diluted 1:3 with N-2-hydroxyethylpiperazine-N9-2-ethanesulfonic acid (HEPES, 25 mM, pH 7.4) and vortexed. The solution was centrifuged three times at 2000 × g for 15 min at 4°C. The supernatant containing the crude mucus was centrifuged two times more at 12,000 rpm for 15 min at 4°C and filter sterilized by passage through a 0.45 µm pore size filter (IWAKI, International Medical, Brussels, Belgium) and stored at -80°C. Protein content was determined using a Biorad protein assay kit (Biorad, Nazareth, Belgium).

Minimal inhibitory concentrations of medium-chain fatty acids against *C. jejuni*

MIC values of MCFA against *C. jejuni* were determined using a microdilution method following the Clinical and Laboratory Standards Institute guideline M31-A3 (CLSI, 2008) with some minor modifications. MIC values of MCFA were determined in triplicate at pH 6.0 and pH 7.5 using HCl or NaOH to obtain the desired pH-values. Caproic (C_6:0), caprylic (C_8:0) and capric (C_{10}:0) acid (all products from Sigma, St. Louis, MO) were tested after serial 2-fold dilutions in 96 microtiter well plates in Mueller-Hinton (MH) broth (CM0405; Oxoid), without the addition of lysed horse blood, ranging from 0.063 to 16 m*M*. These tested concentrations were chosen based on preliminary studies (data not shown) conducted in our laboratory. Where needed, acids were fully dissolved into the broth by heating (56°C). Inoculum was prepared by collecting colonies directly from blood agar plates that were incubated for 24 to 48 h at 42°C in a microaerobic environment, suspending them into sterile PBS until a turbidity equivalent to a 0.5 McFarland standard was achieved, and diluting 1:4 in MH broth. Five µL of this suspension was inoculated into 195 µL medium in each well, so the final inoculum contained approximately 10^5 cfu/mL. These suspensions were incubated for 24 h at 42°C in a microaerobic environment (as above) after which microbial growth was assessed. Where needed, plates were incubated for an additional time until growth was observed for the positive controls. The lowest concentration completely inhibiting visible growth was considered the MIC.

Time-kill curve experiments

Time-kill curve experiments were conducted in MH broth supplemented with the MCFA caproic, caprylic, or capric acid at 1, 4, and 16 m*M* or without acid (growth control). The solutions were brought to pH 6.0 using HCl or NaOH followed by inoculation with a *C. jejuni* suspension grown for 17 h in NB2 with supplements, so the *C. jejuni* concentration in these solutions was approximately 10^7 cfu/mL. Bacterial counts were determined at 0, 1, 2, 4, 8, 16, and 24 h post-inoculation (pi). The experiment was conducted in triplicate.

Time-kill curve experiments in HBSS supplemented with 5 mg/mL chicken intestinal mucus and capric acid at 1, 4, and 16 m*M* or without capric acid (growth control), were conducted analogously.

Supplementation of non-coated medium-chain fatty acids to broilers experimentally inoculated with *C. jejuni*

Day-of-hatch broiler chicks (n = 50) were raised in group. At the age of 15 d, all chicks were orally inoculated with approximately 5×10^7 cfu of tylosin-sensitive *C. jejuni* strain KC 40. At 23 days of age, the chicks were randomly assigned to 5 treatment groups (n = 10 per group): negative control (*Campylobacter* infected, no MCFA); positive treatment control (*Campylobacter* infected, 1% (wt/wt) tylosin granules (TYLAN 100 Granules, Elanco, Brussels, Belgium)); and 3 acid treatment groups provided with 1% (wt/wt) non-coated caproic, caprylic, or capric acid sodium salt (all products from Sigma) in the feed. From this moment, chickens were housed in groups in separate isolating chambers. Acids and tylosin granules were added for the last 72 h of the 28-day trial. Equal amounts of feed were provided for each group during treatment and care was taken that all animals had unlimited access to the feed. After treatment the chicks were euthanized by injection of T61 IV in the wing vain (600 µL/chick), and the ceca as well as their contents were collected for *C. jejuni* enumeration (see below).

Supplementation of coated medium-chain fatty acids to broilers experimentally inoculated with *C. jejuni*

An *in vivo* trial with coated MCFA was performed analogously to the experiment with non-coated MCFA. The chicks were randomly assigned to 4 treatment groups (n = 10 per group): negative control (*Campylobacter* infected, no MCFA); tylosin-control (*Campylobacter* infected, 1% tylosin granules); and 2 acid treatment groups provided with coated caproic or caprylic acid (all products from Sanluc International nv, Oosterzele, Belgium.), at final concentrations of the pure acids in the feed of 1% (wt/wt). Coated capric acid could not be obtained for this study.

Cecal loop model

A ‘cecal loop’ model was used to examine the effect of the sodium salt of capric acid on cecal *Campylobacter* numbers. This model allows direct comparison of *C. jejuni* numbers in both ceca of the same animal. One cecum (positive control) is injected with a control solution, while in the other cecum a test compound is injected (Van Deun *et al.*, 2008b). Day-of-hatch broiler chicks (n = 6) were raised in group. At the age of 14 days, chicks were divided in two groups (n = 3) and housed in separate isolation units. At the age of 15 days, all chicks of group 1 were inoculated with approximately 3.5×10^7 cfu/mL of *C. jejuni* strain KC 40, while

the chicks of group 2 were kept in a *Campylobacter* free environment. At the age of 21 days, cecal loops were applied in all six animals. Twelve h before surgery, buprenorfine was administered. Chicks were anaesthetized with isofluran and the abdominal cavity was opened with an incision of two inch, caudal of the sternum. The ceca were exposed and one loop in each cecum was constructed using Vicryl 4/0 surgical suture. One of the ceca of each animal of group 1 was injected with 200 µL 75 m*M* sodium caprate (Sigma) in HBSS, while the other cecum was injected with 200 µL HBSS, and thus served as control. In the animals of group 2, one cecum was injected with 100 µL 40 m*M* sodium caprate in HBSS, while the other cecum was injected with 100 µL HBSS. After this, both ceca were injected with 100 µL of a *C. jejuni* KC 40 suspension of approximately 2×10^5 cfu/mL in HBSS.

After injection, the cecum was repositioned in the abdominal cavity and the peritoneum, muscles and skin were sutured. After 24 h the chickens were euthanized by intravenous injection with T61 and the ceca with their contents were collected for cecal *Campylobacter* enumeration (see below).

Cecal *Campylobacter* enumeration

Ceca and contents were cut into little fragments, weighed, and diluted 1:9 (wt/vol) in NB2 with supplements. After homogenization, a 10-fold dilution series was made in HBSS. Of each dilution, 100 µL was spread on mCCDA plates. After 22 h incubation at 42°C under microaerobic conditions, colonies were counted. For enrichment, diluted cecal samples in NB2 were incubated at 37°C under microaerobic conditions. After 24 h, samples were plated on mCCDA and incubated at 42°C in a microaerobic environment. After 24 and 48 h plates were examined for the presence or absence of *C. jejuni*.

Statistical analysis

Data of the *in vivo* trials and the 'cecal loop' experiment were analyzed by SPSS 17.0 software for Windows. The significance level α was set at 0.05. *Campylobacter* counts were first transformed to $\log_{10}$ counts before statistical analysis. A one-way analysis of variance (ANOVA) was then carried out to compare the means of $\log_{10}$ transformed counts in chicken cecal contents of all groups (treated groups and control group) in the *in vivo* trials. Significant differences were assessed by Bonferroni Post Hoc tests. *P*-values below 0.05 were considered significantly different. A paired-samples *t*-test was performed to compare the means of $\log_{10}$ transformed counts in chicken cecal contents of the treated group and control group in the 'cecal loop' experiment. *P*-values below 0.05 were considered significantly different.

RESULTS

Minimal inhibitory concentrations of medium-chain fatty acids against *C. jejuni*

Table 1.1.1 shows the results of the determination of the MIC values of the MCFA caproic, caprylic, and capric acid for two poultry and four human derived *C. jejuni* strains.

Table 1.1.1 Overview of the minimal inhibitory concentration (MIC) of caproic, caprylic and capric acid, tested in Mueller-Hinton (MH) broth at pH 6.0 and 7.5 against 2 chicken and 4 human derived *Campylobacter jejuni* strains

	Number of strains with a MIC of (mM)								
Compound	0.063	0.13	0.25	0.5	1	2	4	8	16
Caproic acid									
pH 6.0	-	1	5	-	-	-	-	-	-
pH 7.5	-	-	-	-	-	3	3	-	-
Caprylic acid									
pH 6.0	-	-	1	5	-	-	-	-	-
pH 7.5	-	-	-	-	-	2	4	-	-
Capric acid									
pH 6.0	-	-	1	5	-	-	-	-	-
pH 7.5	-	-	-	-	-	3	3	-	-

MIC value variation for each strain for a given acid was maximally one 2-fold dilution. The MIC of all fatty acids was 4- to 16-fold lower at pH 6.0 compared to pH 7.5 and was comparable for all three MCFA.

Time-kill curves of medium-chain fatty acids against *C. jejuni*

Based on the MIC values obtained in the previous experiment, time-kill curves were prepared to analyze the bactericidal effect of MCFA toward *C. jejuni* strain KC 40 using 1, 4 and 16 m*M* at pH 6.0, over a 24-h period. MH broth without acids and adjusted to pH 6.0 served as a growth control.

Colony count variation at each time point for each concentration, between triplicate experiments, was less than 10%. All three MCFA at 1 m*M* were only moderately bactericidal (< 2 logs kill at any time point) toward *C. jejuni* (**figure 1.1.1**). After 24 h, bacterial counts approached or exceeded the initial inocula but were lower than the growth control counts for

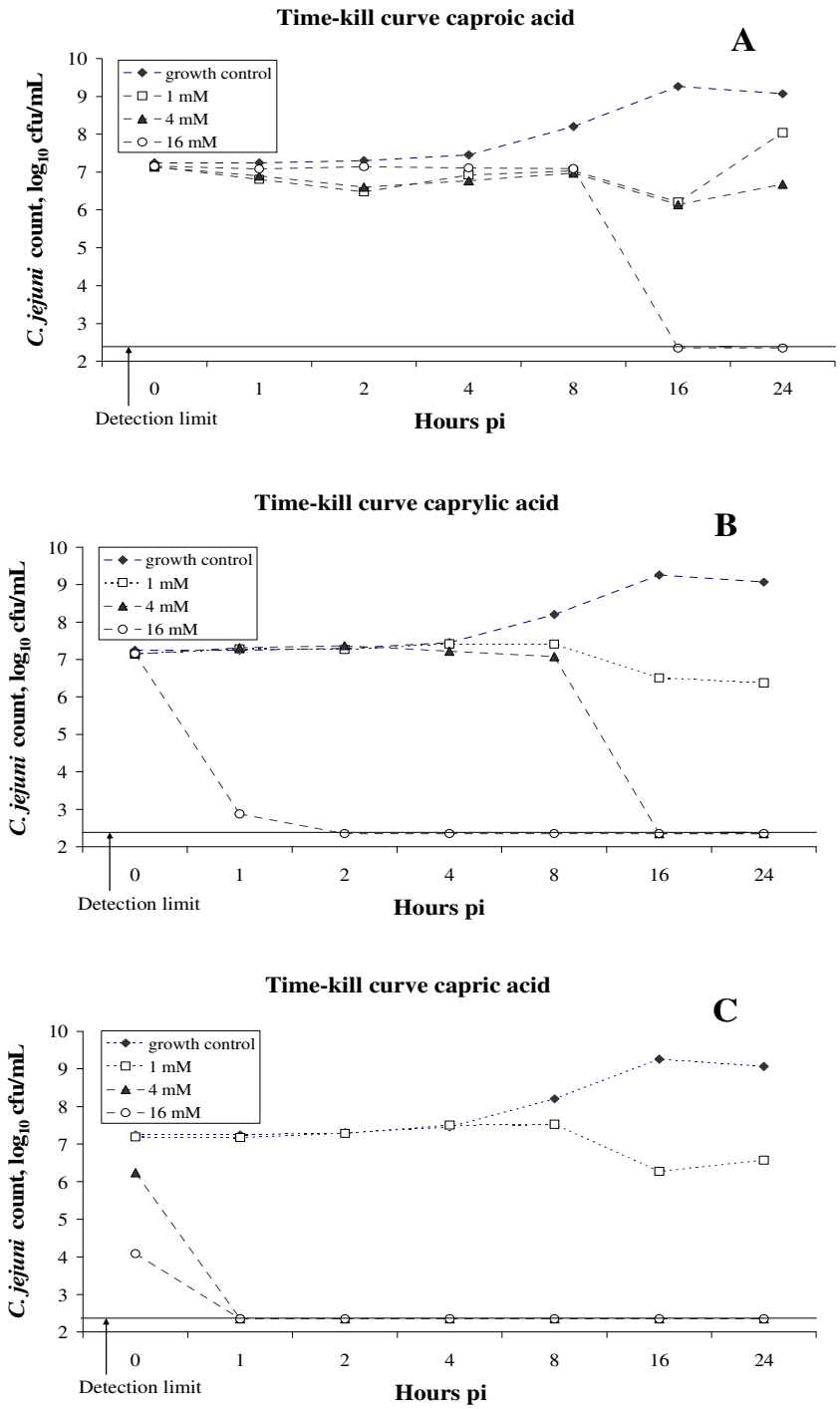

Figure 1.1.1 Time-kill curves of medium-chain fatty acids (MCFA) at pH 6.0 against *Campylobacter jejuni* strain KC 40. (A) caproic acid, (B) caprylic acid, and (C) capric acid. Bacterial counts are represented as $\log_{10}$ cfu/mL. ♦, growth control; □, MCFA at 1 m*M*; ▲, MCFA at 4 m*M*; ○, MCFA at 16 m*M*. The experiment was repeated 3 times, and the average numbers are shown.

all acids. The same result is observed for caproic acid at 4 m*M* (< 1 log kill at any time point). At 4 m*M*, caprylic acid killed all *C. jejuni* bacteria within 16 h after exposure, whereas capric acid killed all bacteria within 1 h pi at this concentration. At 16 m*M*, all three MCFA reduced

bacterial counts below the detection limit (within 16 h pi for caproic acid and within 1 h pi for caprylic and capric acid) and maintained these numbers after 24 h. At 4 and 16 m*M*, capric acid was bactericidal already within seconds, as proven by strongly reduced bacterial numbers shortly (at 0 h) after exposure.

Feed supplemented with coated or non-coated medium-chain fatty acids does not reduce cecal *C. jejuni* colonization in broilers

Feed supplemented with non-coated (i.e. free) sodium caproate, caprylate or caprate did not significantly ($P > 0.05$) reduce cecal *Campylobacter* load in chickens, compared with the feed without supplements (**figure 1.1.2**). Cecal *Campylobacter* counts of animals fed tylosin-supplemented feed was again significantly ($P < 0.05$) reduced by approximately 3.5 logs, compared to the control group (3.9 ± 0.7 and 7.4 ± 0.6 $\log_{10}$ cfu/g, respectively). Feed uptake (data not shown) was similar for all groups during the treatment period.

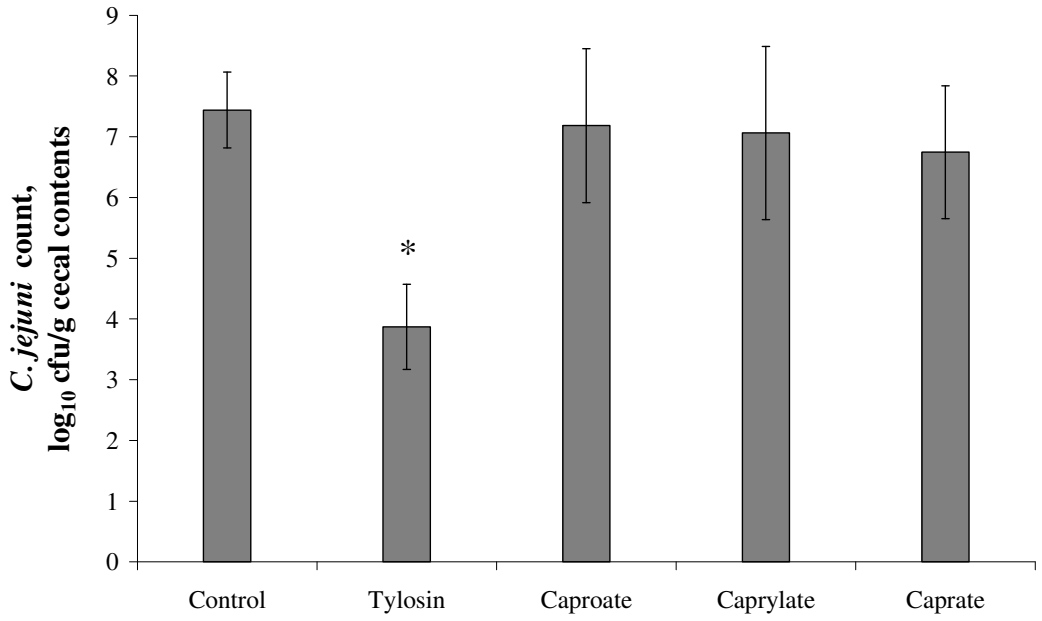

Figure 1.1.2 Cecal *Campylobacter jejuni* counts in 27-day-old broilers (n = 10 chickens/treatment) fed un-supplemented feed, tylosin, and sodium caproate, caprylate, or caprate from 3 days before euthanization. Values are represented as $\log_{10}$ cfu/g cecal contents and are mean ± standard deviation (n = 10). An asterisk denotes significantly lower cecal *Campylobacter* levels compared to the control group ($P < 0.05$).

Feed supplemented with coated caproic or caprylic acid did not significantly ($P > 0.05$) alter cecal *Campylobacter* counts in broilers, compared with the un-supplemented feed (**figure 1.1.3**). Cecal *Campylobacter* load of chickens fed tylosin-supplemented feed was significantly

($P < 0.05$) reduced by approximately 4 logs, compared to the control group (2.8 ± 1.5 and 7.0 ± 0.8 $\log_{10}$ cfu/g, respectively). Feed uptake (data not shown) was similar for all groups during the treatment period.

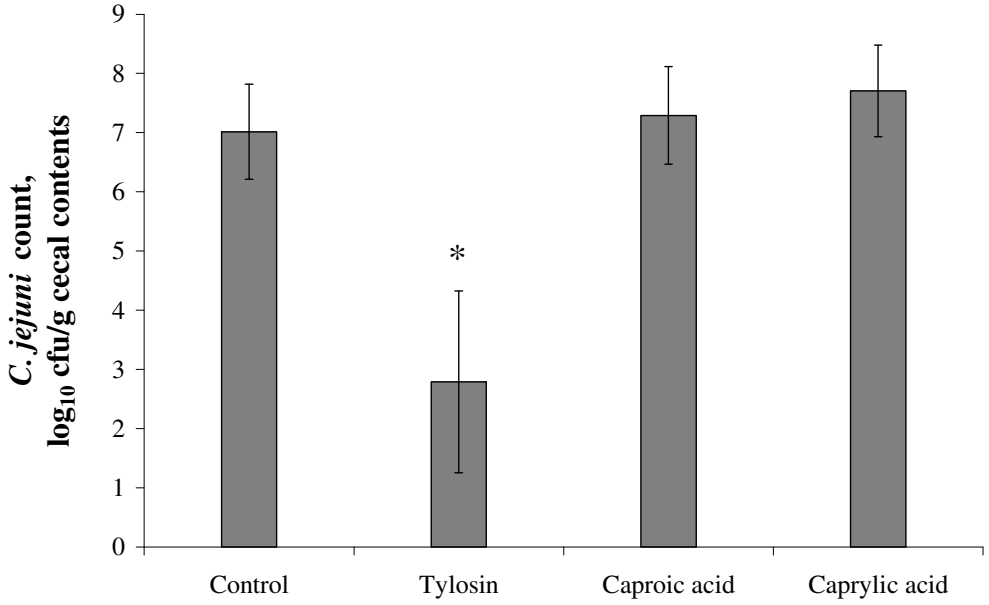

Figure 1.1.3 Cecal *Campylobacter jejuni* counts in 27-day-old broilers (n = 10 chickens/treatment) fed un-supplemented feed, tylosin, and coated caproic or caprylic acid from 3 days before euthanization. Values are represented as $\log_{10}$ cfu/g cecal contents and are mean ± standard deviation (n = 10). An asterisk denotes significantly lower cecal *Campylobacter* levels compared to the control group ($P < 0.05$).

Sodium caprate is not able to reduce cecal *C. jejuni* numbers in the 'cecal loop' model

The cecal content of 21-day-old broilers was estimated to be 1.0–2.0 mL. By injecting 100 µL 40 m*M* (as well as 100 µL *Campylobacter* suspension) or 200 µL 75 m*M* sodium caprate, final caprate concentrations in these ceca ranged then from 1.8–3.3 m*M* or 6.8–13 m*M*, largely sufficient to cause a growth-inhibitory, respectively bactericidal effect toward *C. jejuni* under *in vitro* conditions (see above). Despite these concentrations being present, sodium caprate did not significantly ($P > 0.05$) alter the mean cecal *Campylobacter* levels in the cecal loops of colonized (B) or non-colonized (A) broilers compared with the control cecum after 24 h (**figure 1.1.4**). There was some variability in the results however, with one bird in each study showing an approximate 2 log reduction in cecal *C. jejuni* counts, while in the other two birds there was a slight increase (or decrease), so that overall no significant effect of the treatments could be observed.

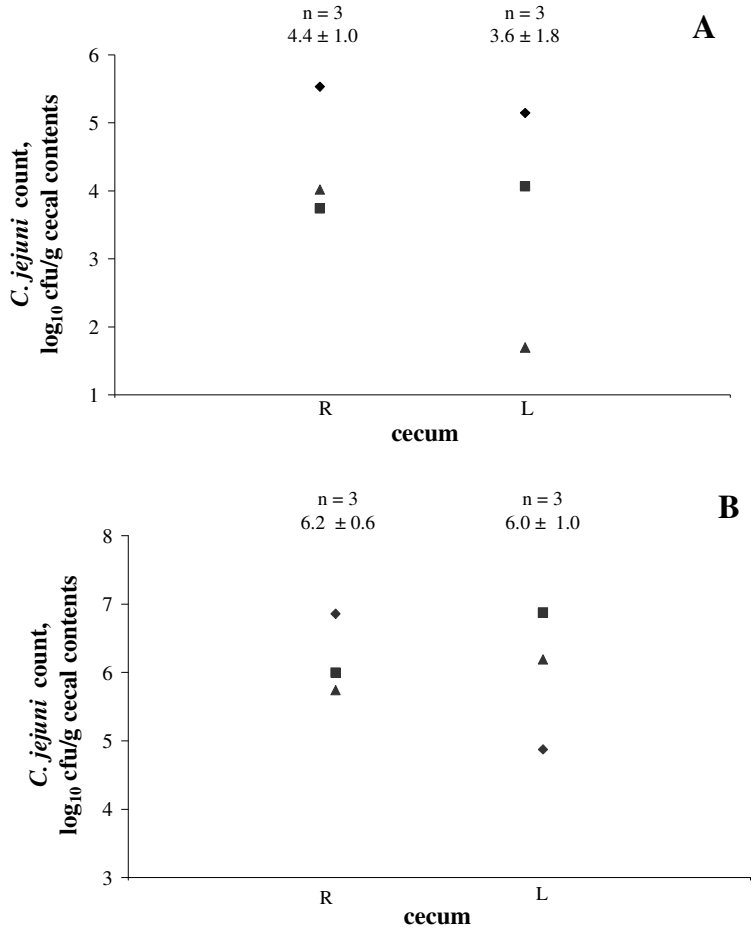

Figure 1.1.4 *Campylobacter jejuni* counts in cecal loops of 22-day-old broilers (n = 3 chickens/treatment). In (A), one cecum of a *Campylobacter*-free chick was injected with 100 µL 40 m*M* sodium caprate (L), the other with 100 µL HBSS (R). Both ceca were injected with 100 µL of a 2 × 10^5 cfu/mL *C. jejuni* suspension and *C. jejuni* counts were determined 24 h later. In (B), one cecum of a *C. jejuni*-infected chick was injected with 200 µL 75 m*M* sodium caprate (L), the other with 200 µL HBSS (R) and *C. jejuni* counts were determined 24 h later. Values are represented as $\log_{10}$ cfu/g cecal contents. Also the mean ± standard deviation (n = 3) is given. Each symbol represents *C. jejuni* counts in both ceca of the same bird.

Chicken intestinal mucus protects *C. jejuni* against anti-*Campylobacter* activity of capric acid

Figure 1.1.5 shows the influence of chicken intestinal mucus on growth and survival of *C. jejuni* KC 40 in the presence of capric acid at pH 6.0. Bacterial counts were determined at different time intervals in MH broth (A) or in HBSS with 5 mg/mL chicken intestinal mucus (B). MH broth, respectively HBSS with 5 mg/mL mucus at pH 6.0 without capric acid served as the growth controls.

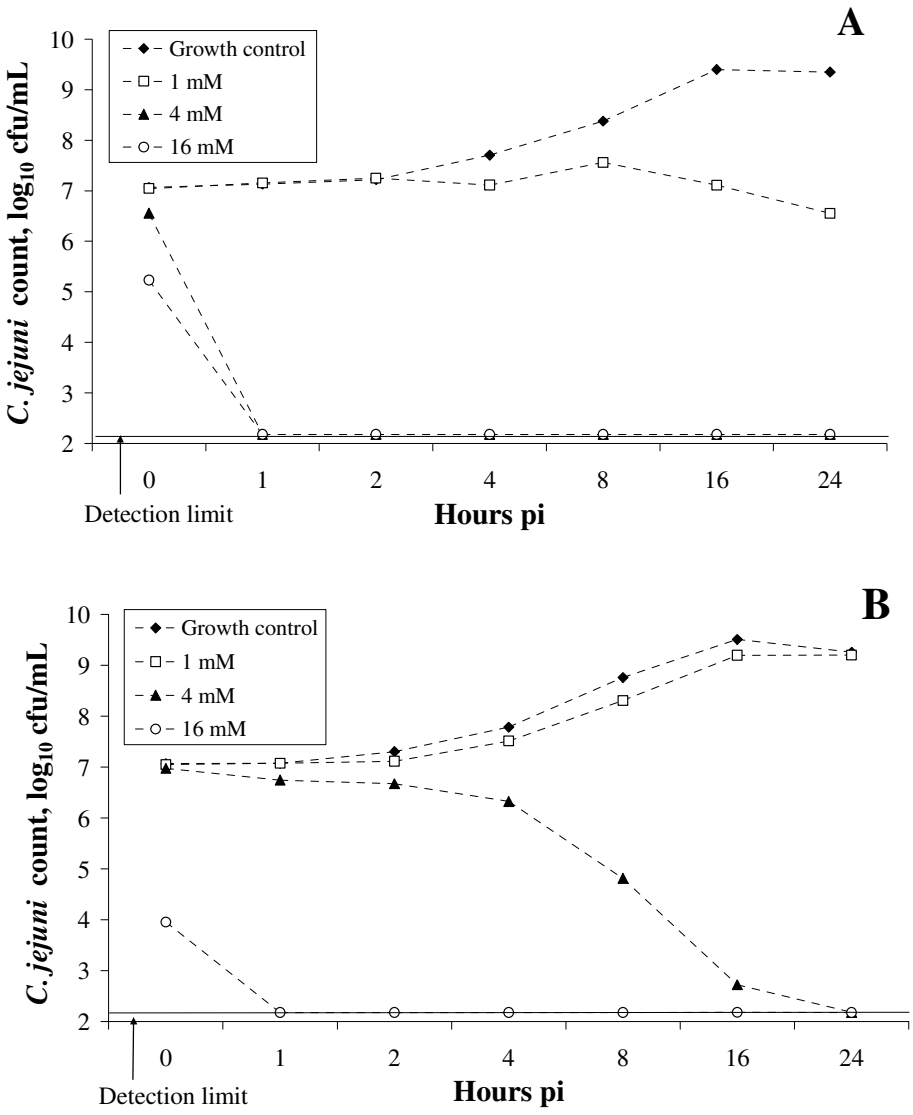

Figure 1.1.5 Influence of chicken intestinal mucus on growth and survival of *Campylobacter jejuni* KC 40 in the presence of capric acid at pH 6.0. Time kill curves are shown for capric acid in (A) MH broth, and (B) HBSS supplemented with 5 mg/mL chicken intestinal mucus. Bacterial counts are represented as $\log_{10}$ cfu/mL. ♦, growth control; □, capric acid at 1 m*M*; ▲, capric acid at 4 m*M*; ○, capric acid at 16 m*M*. The experiment was repeated 3 times, and the means are shown.

Colony count variation at each time point for each concentration, between triplicate experiments, was less than 10%. Mucus had no effect on the bactericidal properties of capric acid at 16 m*M*. Bacterial counts were reduced under detectable levels within the hour whether (B) or not (A), mucus was added. Also capric acid at 4 m*M* killed all bacteria within the hour without mucus (A), while this reduction was only obtained after 24 h in the presence of mucus (B). At 1 m*M* capric acid was growth-inhibitory toward *C. jejuni* in the absence of mucus (A), but not when mucus was added (B).

DISCUSSION

The first objective of this study was to evaluate the antimicrobial activity of MCFA against *Campylobacter jejuni in vitro*. MIC values of MCFA toward *C. jejuni* were pH-dependent, probably attributable to the increased dissociation of the acids in neutral environments (Davidson, 1997). This was already reported to be the case for *C. jejuni* (Skrivanova *et al*., 2007) as well as for other bacteria (Sun *et al*., 1998; Boyen *et al*., 2008). Although the mode of action of MCFA and fatty acids in general on *C. jejuni* is still uncertain, the acids probably act by diffusing into bacterial cells in the un-dissociated form where they later on dissociate in the near-neutral environment of the bacterial cytoplasm, as hypothesized by Sun *et al*. (1998) for butyric acid. These accumulating anions lead to intracellular acidification, which can eventually cause killing of the bacterium (Russell, 1992; Sun *et al*., 1998). The observed MIC values of MCFA were at least 10-fold lower compared to the MIC of the short-chain fatty acid (SCFA) butyrate (6.25 m*M* in Brain Hart Infusion broth) as determined by Van Deun *et al*. (2008a) against *C. jejuni* KC 40 at pH 6.0. Lower MIC values for MCFA compared to SCFA can also be observed for *Salmonella* Typhimurium (Boyen *et al*., 2008), as well as for several other Gram-positive and -negative bacteria (Nakai & Siebert, 2003). Time-kill curve experiments revealed pronounced bactericidal properties of MCFA toward *C. jejuni* at pH 6.0, which approaches the mean cecal pH of broilers during their life-cycle (van der Wielen *et al*., 2000), with concentrations of 4 m*M* being able to reduce bacterial numbers under detectable levels after 16 h for caprylic and capric acid. The same result was achieved for caproic acid at 16 m*M*. At this concentration, caprylic and capric acid are strongly bactericidal within minutes. This is in accordance with Thormar *et al*. (2006), who showed that a 10-min incubation period in 10 m*M* caprylic and capric acid in aerobic broth at 37°C decreased *C. jejuni* counts by approximately $\log_{10}$ 3.5 and $\log_{10}$ 7, respectively (pH unreported). Also Skrivanova *et al*. (2007) found similar results: a 10-min incubation period in 1 mg/mL capric acid (≈ 5.8 m*M*) reduced *C. jejuni* levels from > 10^9 cfu/mL below the detection limit, both at pH 4.5 and 7.0. Hence, our results show a profound bactericidal, dose-dependent activity of the tested MCFA toward *C. jejuni in vitro* which may indicate a promising role for these compounds as possible agents to reduce cecal *C. jejuni* counts in colonized broiler chickens.

Therefore, our second objective was to test the antimicrobial activity of MCFA against *C. jejuni in vivo*. First, an experiment using free and micro-encapsulated MCFA was performed. Coating MCFA on micro-beads prevents absorption of the acids in the upper tract and results in a slow release of the acids during transport along the gastro-intestinal tract, allowing them

to reach the small intestine and the ceca (Van Immerseel *et al*., 2004b). This is of great importance because the latter represents the predominant site for *Campylobacter* colonization, where the bacterium is found deep in the cecal crypts within the mucus layer (Beery *et al*., 1988). For this reason, only the ceca were considered for analysis. MCFA concentrations of 1% were used in this trial, as Solis de los Santos *et al*. (2008b) found consistent results when using 0.7% or 1.4% caprylic acid. Coated capric acid could not be obtained for this experiment. Coated MCFA-supplemented feed, as well as non-coated MCFA sodium salts did not reduce cecal *Campylobacter* counts in experimentally infected chicks when administered three days before euthanization. Sodium salts (solid at room temperature) of MCFA were used for practical convenience and are believed to react in the same way as their acids (liquid at room temperature, except capric acid), because *in vitro* experiments conducted at our laboratory showed similar bactericidal properties against *C. jejuni* for both formulations (data not shown). Tylosin, a macrolide antibiotic that has been banned for use as a growth promoter by the EU Commission in 1999 (Dibner & Richards, 2005), and used as a positive treatment control, reduced cecal *Campylobacter* levels markedly in both *in vivo* experiments, pointing towards the successful performance of these tests.

For caprylic acid, our results are not in agreement with those obtained by Solis de los Santos *et al*. (2008b, 2009). These authors demonstrated a decrease in cecal *C. jejuni* counts of approximately 3 to 4 logs when supplementing feed with 0.7% caprylic acid 3 days before necropsy, both in 15-day-old (Solis de los Santos *et al*., 2008b) and 42-d-old (market-aged) (Solis de los Santos *et al*., 2009) experimentally infected broilers. Possibly, these contradictory observations could be attributed to 1) a difference in formulation of the acid in the feed, allowing it to reach the ceca at higher concentrations in the experiments of Solis de los Santos and coworkers; or 2) *C. jejuni* strain-specific properties, as we inoculated the chicks with a highly invasive strain that might be less susceptible toward the effects of MCFA in the broiler cecum compared to less invasive strains; or 3) other factors, such as differences in genetic background of the birds used in the 2 studies or differing dietary influences on cecal biochemistry. Next to these contradictory results, also the addition of 0.12% or 0.24% emulsified monocaprin, the 1-monoglyceride of capric acid, to drinking water and feeds of colonized birds was shown to significantly reduce fecal *Campylobacter* counts in these animals with 1 to 2 logs after 2-3 days (Hilmarsson *et al*., 2006). Since monocaprin is probably hydrolyzed into capric acid and glycerol by lipases in the digestive tract (Hilmarsson *et al*., 2006), this reduction is most likely attributed to the bactericidal properties of the former.

To determine whether capric acid, having the highest *Campylobacter* reduction activity *in vitro*, is capable of reducing *Campylobacter* numbers in the ceca of broiler chickens at concentrations that are bactericidal *in vitro*, the sodium salt of capric acid was tested in a cecal loop model. Sodium caprate was used because of its better solubility in aqueous solutions over capric acid, so higher concentrations could be injected in the ceca. Sodium caprate failed however to inhibit the growth of, or to kill *Campylobacter* in the broiler chicken cecum at concentrations that are growth-inhibitory or bactericidal *in vitro*, respectively.

Together these results suggest that *C. jejuni* probably is occupying a protected niche in the ceca of broilers, rendering them less susceptible to the bactericidal effects of capric acid seen *in vitro*. The mucus layer, which covers the entire surface of the chicken intestinal tract and is in fact seen as a part of the innate host response (Forstner *et al*., 1995), definitely plays a role herein since capric acid was less effective in inhibiting the growth of, and killing *C. jejuni* in the presence of chicken intestinal mucus. This was also already observed for the sodium salt of the SCFA butyric acid (Van Deun *et al*., 2008a). Whether this decreased susceptibility is caused by interference between mucosal constituents and the antimicrobial potency of these acids, or an indirect effect on the *C. jejuni* bacteria is not clear. Thus, where the chicken intestinal mucus layer usually is regarded as an anti-bacterial barrier by protecting against luminal microflora (Forstner *et al*., 1995), it seems that in the case of *Campylobacter* it forms a protective and supportive environment making it hard to eradicate this bacterium from the chicken intestinal tract, once colonized.

In conclusion, our results show strong bactericidal properties of MCFA toward *C. jejuni in vitro*, as previously reported. However, under the test conditions applied, MCFA were not effective at decreasing cecal *C. jejuni* counts in experimentally infected broilers and in the cecal loop model due to the protective effect of intestinal mucus. Most likely the concentrations applied were too low to target *C. jejuni* in its protective environment. Another strategy is to prevent *C. jejuni* colonization in broilers by administering MCFA preventively, from day-of-hatch. Indeed, van Gerwe *et al*. (2009) showed recently that the addition of a MCFA mixture to the feed at 1% reduces the probability of broilers to become colonized with *C. jejuni*.

To conclude, the application of MCFA cannot be advocated unambiguously to control *C. jejuni* in broilers until further studies have resolved apparently contradictory results.

Acknowledgements

This work was financed by a grant of the Federal Public Service for Health, Food Chain Safety and Environment (FOD, Brussels, Belgium): project RT08/8-CAMPOUL; and was further supported by Sanluc International nv (Oosterzele, Belgium). The excellent technical assistance during the laboratory analyses by Nathalie Van Rijsselberghe and Roselien Schelfaut was greatly appreciated. Coated fatty acids were kindly provided by Luc Goethals, Sanluc International nv.

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CHAPTER 3: EXPERIMENTAL STUDIES

3.1. EFFICACY OF WATER AND IN-FEED APPLICATIONS OF MEDIUM-CHAIN FATTY ACIDS ON *CAMPYLOBACTER JEJUNI* COLONIZATION AND TRANSMISSION IN BROILER CHICKENS

3.1.2. **Application of medium-chain fatty acids in drinking water increases the *Campylobacter jejuni* colonization treshold in broiler chicks**

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Poultry Science (2012), 91:1733-1738

SUMMARY

Campylobacteriosis is the most reported bacterial-mediated gastro-enteritic disease in many developed countries. Broiler chickens are a natural host for *Campylobacter* spp. and contaminated poultry meat products are a major source for transmitting pathogenic *Campylobacter* strains to humans. Currently, no intervention measure efficiently and effectively controls this pathogen in poultry flocks. Medium-chain fatty acids (caproic, caprylic, capric and lauric acid) show a marked anti-*Campylobacter* activity *in vitro*. However, in recent trials using our *in vivo* models, administering these acids to the feed of broiler chicks did neither prevent, nor reduce cecal *C. jejuni* colonization in broilers. In the present study we examined whether a drinking water application of medium-chain fatty acids might be more effective in combating *Campylobacter* colonization in poultry. Although *Campylobacter* colonization and transmission was not reduced, we demonstrate that adding an emulsion of a mixture of caproic, caprylic, capric and lauric acid to the drinking water of broiler chicks reduces their colonization susceptibility and prevents *C. jejuni* survival in drinking water. Thus, the merit of water applications of medium-chain fatty acids is the reduction of the probability of *Campylobacter* entry into and transmission throughout a flock.

Keywords:

Campylobacter jejuni; broiler chicken; medium-chain fatty acid; water application; colonization treshold

INTRODUCTION

Campylobacteriosis is the most reported zoonotic disease in many developed countries, with in particular *Campylobacter jejuni* as the causative agent (EFSA, 2010a). Broiler chickens are a natural host for this pathogen and their carcasses often become contaminated during slaughter. Poultry products are therefore considered one of the most significant sources for campylobacteriosis in humans (Friedman *et al.*, 2004; Vellinga & Van Loock, 2002; EFSA, 2010b). Hygiene and biosecurity efforts aimed at minimizing contamination of poultry with *Campylobacter* seem not to be effective. Furthermore, no reliable and practical intervention measure is currently available to reduce *Campylobacter* colonization of the broiler gut (Hermans *et al*., 2011a).

For this purpose, the use of medium-chain fatty acids (MCFA) might be promising due to their marked antibacterial activity against *C. jejuni in vitro*, which was especially observed for capric acid. Furthermore, the antibacterial potency of MCFA is believed to exceed that of short-chain fatty acids (SCFA) (Hermans *et al*., 2010). Several recent studies described the evaluation of in-feed SCFA (formic, acetic, propionic and butyric acid) and MCFA (caproic, caprylic, capric and lauric acid) to reduce (Hermans *et al*., 2010; Molatova *et al*., 2010; Solis de los Santos *et al*., 2010) or prevent (Van Deun *et al*., 2008; Solis de los Santos *et al*., 2008) cecal *Campylobacter* colonization in broilers; however, inconsistent results have been reported. Alternatively to their in-feed use, MCFA can be administered through the drinking water as well, but studies using water applications of MCFA are scarce. Although only poorly soluble in aqueous solutions, these acids can be mixed in the drinking water by creating MCFA emulsions (Hilmarsson *et al*., 2006). These emulsions may exert their bactericidal properties on *C. jejuni* in the drinking water, at the crop and in the stomach of the animals, in this way providing a barrier for bacterial entry in the chicken gut. Therefore, the aim of this study was to investigate the effect of a (1:1) commercial MCFA/water emulsion in the drinking water (0.4% vol/vol) of broiler chickens on *Campylobacter* susceptibility of birds, transmission and cecal survival.

MATERIALS AND METHODS

Experimental animals

Day-of-hatch Ross broiler chickens of both sexes from a local farm were raised individually and provided with a commercial starter feed *ad libitum*. Husbandry, euthanasia methods,

experimental procedures and bio-safety precautions were approved by the Ethical Committee (EC numbers 2009/114wi and EC2010/110) of the Faculty of Veterinary Medicine, Ghent University, Ghent, Belgium. Chicks were examined for the presence of *Campylobacter* in mixed fecal samples prior to inoculation and proved to be *Campylobacter* free.

Bacterial strains and culture conditions

Campylobacter jejuni strain KC 40 from poultry origin was used for all experiments. This strain colonizes chickens to a high level (Van Deun *et al.*, 2008; Hermans *et al*., 2010). Bacteria were routinely cultured in Nutrient Broth No.2 (NB2, CM0067; Oxoid Ltd., Basingstoke, Hampshire, UK) supplemented with Modified Preston *Campylobacter*-selective supplement (SR0204E; Oxoid) and *Campylobacter*-specific growth supplement (SR0232E; Oxoid), at 42°C under microaerobic conditions (5% O_2, 5% CO_2, 5% H_2, 85% N_2). *C. jejuni* bacteria were enumerated by preparing 10-fold dilutions in Hank's Balanced Salt Solution (HBSS; GIBCO-BRL, Invitrogen, Carlsbad, CA) and plating on modified charcoal cefoperazone deoxycholate agar (mCCDA, CM0739; Oxoid) supplemented with CCDA selective supplement (SR0155E; Oxoid) and *Campylobacter*-specific growth supplement (SR0232E; Oxoid), followed by microaerobic incubation at 42°C for 22 h.

Anti-*C. jejuni* effect of the MCFA emulsion *in vitro*

The effect of a commercial MCFA emulsion (delivered by Vitamex, Drongen, Belgium), containing approximately 50% MCFA (a mixture of caproic, caprylic, capric and lauric acid), on *C. jejuni* strain KC 40 survival was assessed (Deschepper *et al*., 2003). The MCFA emulsion was diluted in tap water to a final concentration of 0.4% (vol/vol), the dose recommended by the manufacturer. Tap water without MCFA was used for control. Water samples were inoculated with a *C. jejuni* suspension grown for 17 h, so the final starting inoculum was approximately 10^7 cfu/mL, and incubated at 25°C under aerobic conditions. After 1 minute of incubation *C. jejuni* counts were enumerated.

Effect of the MCFA emulsion on established *C. jejuni* colonization in four-week-old broiler chicks

Twenty-four chicks were raised in group and received a commercial starting feed and drinking water *ad libitum*. The animals remained on the same feeding and drinking regime for the entire duration of the experiment except for the last three days. After two weeks all animals were orally challenged with approximately 10^6 cfu *C. jejuni* KC 40. At 25 days of

age, birds were assigned to two treatment groups (n = 12 birds/group). Animals of group 1 received standard drinking water, while those of group 2 received water supplemented with 0.4% (vol/vol) MCFA emulsion until the end of the experiment (trial 1). After four weeks all birds were euthanized by injection of T61 IV (Intervet, Belgium) in the wing vein, and the ceca as well as their contents were collected for *Campylobacter* enumeration (see below).

Effect of the MCFA emulsion on *Campylobacter* colonization susceptibility of individually housed broiler chicks

Day-of-hatch broiler chicks (n = 60) were housed individually and randomly assigned to six groups. Animals of groups 1 to 3 received standard drinking water, while those of groups 4 to 6 received water supplemented with 0.4% (vol/vol) MCFA emulsion. All animals received a commercial starting feed *ad libitum* and animals remained on the same feeding and drinking regime for the entire duration of the experiment. At 14 days of age, all birds were orally inoculated with 1 mL of 3×10^2 (groups 1 and 4), 2×10^3 (groups 2 and 5) or 2×10^4 (groups 3 and 6) cfu/mL of *C. jejuni* KC 40 (trial 2). Twenty-four h after inoculation the chicks were euthanized and the ceca as well as their contents were collected to examine the *C. jejuni* status of the animals (see below).

Effect of the MCFA emulsion on *C. jejuni* transmission in broiler chicks

Day-of-hatch broiler chicks (n = 60) were randomly assigned to six groups (n = 10 birds/group). Animals of groups 1 to 3 received standard drinking water, while those of groups 4 to 6 received water supplemented with 0.4% (vol/vol) MCFA emulsion (trial 3). All animals received a commercial starting feed *ad libitum*. The animals remained on the same feeding and drinking regime for the entire duration of the experiment. At 14 days of age, three out of ten birds per group were orally inoculated with ca. 3×10^3 cfu *C. jejuni* KC 40. Five days after inoculation, all birds were euthanized and the ceca as well as their contents were collected for *Campylobacter* enumeration (see below). Starting after inoculation, daily drinking water samples were taken from all groups and examined for the presence of *C. jejuni* bacteria.

Determination of *Campylobacter* status and cecal counts

Ceca and contents were cut into little fragments, weighed, and diluted 1:9 (wt/vol) in NB2 with supplements. After homogenization, samples were serially diluted and 100 µL of each dilution was spread on mCCDA plates. After 24 h incubation at 42°C under microaerobic

conditions, plates were examined for the presence of specific *Campylobacter* colonies and/or enumerated. Bacterial counts in broiler drinking water (with our without the addition of 0.4% (vol/vol) MCFA emulsion) samples were analyzed analogously.

Statistical analysis

Data of all *in vivo* trials were analyzed by SPSS 17.0 software for Windows. The significance level α was set at 0.05. A Pearson chi-square test was performed to analyze the data of *in vivo* trial 2. For *in vivo* trials 1 and 3 *Campylobacter* counts were first transformed to $\log_{10}$ counts before statistical analysis. Next, a one-way analysis of variance (ANOVA) was then carried out to compare the means of $\log_{10}$-transformed counts in chicken cecal contents of all groups (MCFA-treated groups and control groups) in the *in vivo* trials. Significant differences were assessed by Bonferroni Post Hoc tests. *P*-values below 0.05 were considered significantly different.

RESULTS AND DISCUSSION

Recent research revealed marked anti-*Campylobacter* activity *in vitro* for the medium-chain fatty acids (MCFA) caproic, caprylic and capric acid (Hermans *et al*., 2010). However, neither upon feeding these acids to broiler chicks, nor upon direct injection of their salt solutions into the ceca, *Campylobacter* counts were reduced. We therefore concluded that in-feed MCFA or their water-soluble salts are not capable of targeting *Campylobacter* colonization in chicks once the bacterium reaches the ceca. To assess whether water applications of MCFA might be more effective we performed a number of trials where a MCFA emulsion was administrated to the broiler drinking water. Such applications are more ergonomic and because drinking water of broilers is believed to play a key role in transmitting the bacterium through the flock, treating the water with MCFA might reduce *Campylobacter* transmission between birds of the same group (Chaveerach *et al*., 2004; Messens *et al*., 2009; Hermans *et al*., 2011b). In this study we examined the anti-*Campylobacter* effects of a commercial MCFA-in-water (1/1) emulsion (containing caproic, caprylic, capric and lauric acid) at a 0.4% (vol/vol) dose. The influence of the emulsion on *Campylobacter* survival in treated drinking water and in the ceca of colonized birds was assessed. In addition, the effect on the susceptibility for *Campylobacter* colonization as well as on *Campylobacter* transmission between chicks was analyzed. Treating the drinking water used in these trials

with the MCFA emulsion at 0.4% (vol/vol) reduced the pH from 6.9 to 5.4, and thus the bactericidal potency of the acids (Hermans *et al*., 2010).

First, in trial 1, we assessed the effect of the MCFA emulsion on cecal *Campylobacter* counts of already colonized broiler chickens (**figure 1.2.1**). The results showed no significant ($P > 0.05$) differences in cecal bacterial counts of birds receiving treated or control water. These results are in line with our previous observations using in-feed coated MCFA. Other studies showed only a minor reduction in *Campylobacter* counts on cloacal swabs in therapeutic applications of monocaprin, while cecal *Campylobacter* counts were not significantly reduced. In addition, the spread of artificially infected to non-infected birds was not prevented (Hilmarsson *et al*., 2006). Metcalf *et al*. (2011) validated this observation, although in one trial a 3 log reduction in cecal counts was observed after therapeutic administration of 0.175% (wt/vol) caprylic acid to the water.

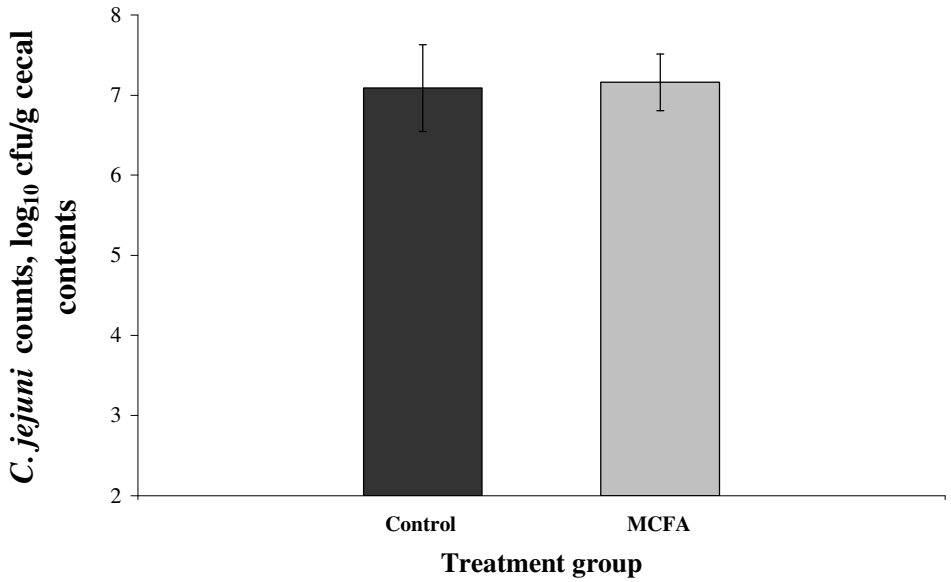

Figure 1.2.1 Cecal *Campylobacter* counts of four-week-old broiler chickens (n = 12) inoculated with approximately 10^6 cfu *C. jejuni* strain KC 40 at two weeks of age and receiving control drinking water (▉) or drinking water supplemented with 0.4% MCFA emulsion (▉). Bacterial numbers were log-transformed and represented as the mean ± standard deviation.

This raised the question whether MCFA are capable to reduce the susceptibility of and prevent *Campylobacter* colonization in chicks by exerting a bactericidal effect in the crop and stomach of the animals. For this purpose, in a next trial, we examined the effect of the MCFA emulsion on the susceptibility of individually housed two-week-old broilers for *C. jejuni* colonization. Birds were inoculated with *C. jejuni* doses of 3×10^2 - 2×10^4 cfu per bird and

euthanized 24 h after inoculation. This time-frame allows *C. jejuni* to establish a high and stable colonizing population in the ceca of the chicks (Smith *et al*., 2008; unpublished data). Our results, presented in **table 1.2.1**, show that less broilers were colonized with *Campylobacter* in groups receiving MCFA-supplemented drinking water compared to groups receiving standard drinking water. These results are in line with the observed increase in colonization treshold using in-feed MCFA applications (van Gerwe *et al*., 2010). In our trial, a *C. jejuni* dose of 2×10^3 cfu was sufficient to colonize 60% of the birds receiving control water, while none of the ten birds receiving MCFA-supplemented drinking water were colonized in their ceca 24 h after inoculation. This statistically significant ($P = 0.03$) difference indicates that broilers receiving MCFA-supplemented drinking water from day-of-hatch onwards should be less prone to cecal colonization with *C. jejuni* compared to birds receiving un-supplemented water.

Table 1.2.1 Colonization status of 15-day-old broilers 24 hours after inoculation with *C. jejuni* strain KC 40

Treatment group	Dose *C. jejuni* KC 40	Number of colonized birds
CDW	-	0/4
CDW	2.8×10^2	2/10
	2.2×10^3	6/10
	2.5×10^4	5/10
MSDW	2.8×10^2	0/10
	2.2×10^3	0/10
	2.5×10^4	1/10

CDW = control drinking water; MSDW = medium-chain fatty acid-supplemented drinking water at a 0.4% (vol/vol) dose. Colonization status is represented as the number of *Campylobacter*-colonized birds out of 10 inoculated birds

Finally, in trial 3 chicks were raised in six groups of ten birds each and were given either MCFA- or un-treated drinking water. Based on the results obtained in trial 2, three out of ten birds were orally inoculated with approximately 3×10^3 cfu of *C. jejuni*. Five days after inoculation of seeders all birds were euthanized and cecal bacterial counts were determined (**figure 1.2.2**). Differences observed between MCFA-treated and control repeats were statistically significant but small, due to a higher variability in bacterial counts in the MCFA-treated group (for individual values, see **appendix A**), the reason of which is unclear. However, it is clear that overall MCFA supplementation to drinking water did not result in an

overall significant ($P > 0.05$) reduction in the cecal *Campylobacter* load of these animals compared to birds receiving control water. This indicates that rapid bird-to-bird transmission mainly happened by the fecal-oral route, because no viable bacteria were recovered from the MCFA-treated water during the entire treatment period, while control water samples from all groups tested positive for *Campylobacter* already two days after inoculation of the seeder birds (results not shown). *In vitro* testing confirmed this observation (results not shown) with a 0.4% (vol/vol) dose of the MCFA emulsion reducing *C. jejuni* counts in drinking water to undetectable levels within the minute.

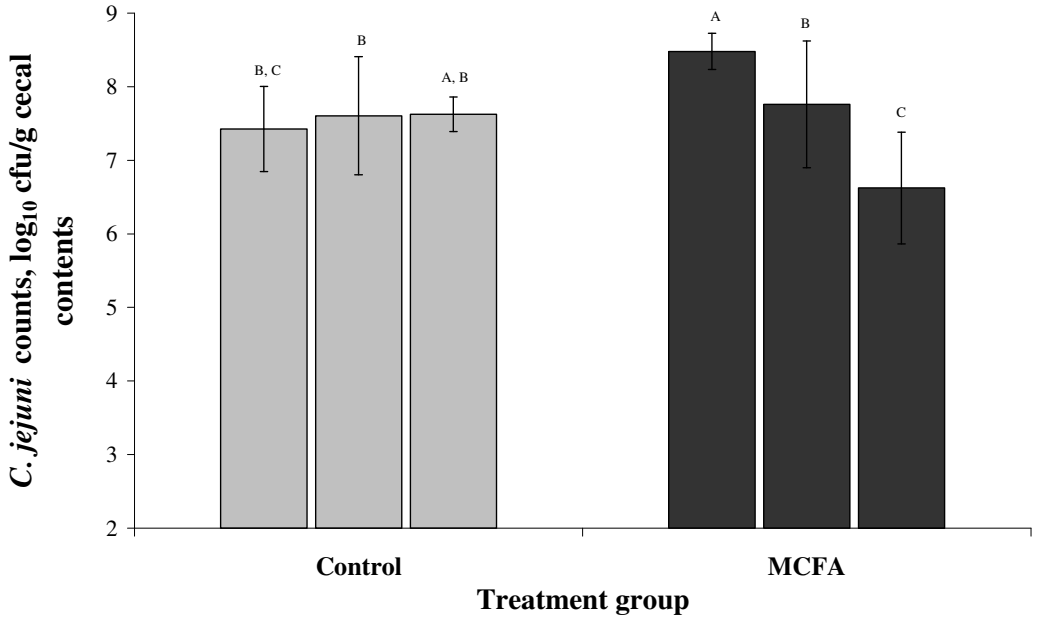

Figure 1.2.2 Cecal *Campylobacter* counts of twenty-day-old broiler chickens (n = 10 birds/group) receiving control drinking water (▯) or drinking water supplemented with 0.4% MCFA emulsion (▮) from day-of-hatch until the end of the experiment. At 15 days of age, 3 out of 10 birds in each group were inoculated with approximately 2.7×10^3 cfu of *C. jejuni* KC 40. Bacterial numbers were log-transformed and represented as the mean ± standard deviation. The experiment was performed in triplicate. Columns with different letters (A, B and C) denote a statistically significant ($P < 0.05$) difference in *Campylobacter* load for two repeats from a different group (MCFA-treated or control).

Thus, on the one hand supplementing MCFA to the drinking water of broilers does neither reduce nor prevents cecal colonization of these animals with *C. jejuni*. On the other hand, it prevents *C. jejuni* survival in the drinking water and reduces the *Campylobacter* colonization threshold of chicks. Probably, the antibacterial effect of the acids in the crop and stomach of the animals prevents the bacterium to reach and colonize the ceca. Indeed, Byrd *et al*. (2001) observed reduced *Campylobacter* counts in crops of broilers receiving drinking water

supplemented with 0.44% lactic acid. This indicates that supplementing MCFA to the drinking water of broiler chicken flocks throughout the whole rearing period, together with proper implementation of biosecurity measures (to limit *Campylobacter* exposure) could be helpful in preventing these flocks to become colonized. Indeed, Chaveerach *et al*. (2004) demonstrated a reduction in cecal *Campylobacter* counts of chicks receiving acidified drinking water only when birds were inoculated with a rather low inoculum concentration (~10^3 cfu). This strategy is likely more effective as a preventive measure for combating *Campylobacter* in poultry compared to administering MCFA after cecal colonization has already been established. Moreover, such an application would be more ergonomic compared to administering the acids to the feed, although also in-feed applications have been reported to be effective in reducing the probability of broilers to become colonized with *C. jejuni* (Skånseng *et al*., 2010, van Gerwe *et al*., 2010). However, the rapid killing of *C. jejuni* in MCFA-treated water at the applied doses at least excludes drinking water as a possible way for *C. jejuni* to enter and colonize a flock, an additional advantage over in-feed addition of MCFA. Indeed, drinking water equipment contaminated with *C. jejuni* is a possible contamination source for broiler chicks (Hermans *et al*., 2011b). Moreover, it has been found in longitudinal studies on commercial broiler flocks that *C. jejuni* is rapidly transmitted in the flock with drinking water as a vector (Herman *et al*., 2003; Messens *et al*., 2009). In any case, it seems that the most effective way to benefit from the antibacterial effects of MCFA seems to be their preventive administration to reduce *Campylobacter* susceptibility of broilers and therefore the probability of *Campylobacter* to enter a flock, but further studies are needed to validate this hypothesis for three reasons. (1) In trial 2, birds were housed individually and only exposed to (inoculated with) *Campylobacter* once, while in commercially reared flocks birds are under a constant contamination pressure from the surrounding environment and each other (Hermans *et al*., 2011b). (2) At the end of trial 3, all birds were colonized with *Campylobacter* indicating that at least one seeder bird in all groups receiving MCFA-treated water was colonized and transmitted *C. jejuni* after inoculation with approximately 3×10^3 cfu of *C. jejuni*, although in trial 2 none of the ten birds orally inoculated with a similar dose (2×10^3 cfu) were colonized after one day. The reason for this discrepancy is not clear and can probably not solely be explained by the slightly higher inoculation dose used in trial 3. A more likely explanation is that *C. jejuni* transmission between the birds in trial 3 was responsible for a reduction in the apparent minimal infective dose (Conlan *et al*., 2011) determined in trial 2, because fecal *Campylobacter* shedding by colonized birds increases the contamination pressure for the remainder of the animals dramatically (Hermans *et al*., 2011b;

unpublished results). Under these conditions, MCFA are not capable anymore to prevent *Campylobacter* colonization of other animals. Therefore, it cannot be ruled out that MCFA supplementation in the drinking water is capable of reducing cecal colonization upon inoculating the seeders with lower inoculum doses. Also, although rather unlikely, it cannot be ruled out that birds negative for *Campylobacter* in their ceca 24 h after inoculation in trial 2 carried the bacterium more proximal in the gastro-intestinal (GI) tract. However, previous infection trials using this *C. jejuni* strain consistently resulted in marked cecal colonization at 24 hours post inoculation of both inoculated as well as sentinel chicks (unpublished results). Based on the results obtained, we suggest that MCFA supplementation induces a reduction in bacterial counts in the proximal GI tract of the animals, resulting in a bacterial population unable to reach and colonize the cecum within 24 hours post-exposure. (3) The degree of *Campylobacter* contamination pressure at which broiler flocks are exposed on the farm can only be speculated upon and depends on the farm that is studied. Therefore, large-scale field studies at broiler chicken farms are needed to determine at what level MCFA supplementation to the drinking water of commercially reared broiler chicks could contribute to the decreased probability of flocks to become colonized with *Campylobacter*.

To conclude, this study demonstrates that a water application of a MCFA emulsion might be successfully applied to prevent *Campylobacter* entrance into a broiler chicken flock by reducing the colonization probability of the birds and excluding drinking water as a potential contamination and transmission source.

Acknowledgements

This work was financed by a grant of the Federal Public Service for Health, Food Chain Safety and Environment (FOD, Brussels, Belgium): project RT08/8-CAMPOUL. The excellent technical assistance of Hanne Vereecke and Nathalie Van Rysselberghe during laboratory analyses was greatly appreciated.

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APPENDIX A

Individual cecal *Campylobacter* counts of twenty-day-old broiler chickens (n = 10 birds/group) receiving control drinking water or drinking water supplemented with 0.4% MCFA emulsion from day-of-hatch until the end of the experiment (results from *in vivo* trial 2). At 15 days of age, 3 out of 10 birds in each group were inoculated with approximately 2.7×10^3 cfu of *C. jejuni* KC 40. Bacterial numbers were log-transformed. The experiment was performed in triplicate.

Control drinking water			Medium-chain fatty acid-treated drinking water		
Repeat 1	**Repeat 2**	**Repeat 3**	**Repeat 1**	**Repeat 2**	**Repeat 3**
7,8	8,4	7,3	8,6	8,3	7,5
7,3	7,8	8,0	8,1	7,7	6,6
6,2	6,1	7,4	8,0	8,3	7,7
8,2	8,2	7,8	8,5	8,6	6,2
7,0	7,3	7,7	8,6	7,3	6,3
7,7	7,9	7,5	8,5	8,5	5,5
7,5	8,4	7,7	8,7	7,3	6,9
7,7	6,3	7,5	8,6	7,0	7,2
7,5	8,0	7,9	8,6	8,6	5,8
†	7,6	7,5	8,6	6,0	†

Underlined: seeder animals

† animal died before end of experiment

CHAPTER 3: EXPERIMENTAL STUDIES

3.2 THE CINNAMON-OIL INGREDIENT *TRANS*-CINNAMALDEHYDE FAILS TO TARGET *CAMPYLOBACTER JEJUNI* STRAIN KC 40 IN THE BROILER CHICKEN CECUM DESPITE MARKED ACTIVITY *IN VITRO*

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Journal of Food Protection (2011), 74:1729-1734

SUMMARY

Campylobacter jejuni is the most common bacterial cause of diarrheal disease in humans worldwide, with poultry products being a major source. Therefore, strategies to decrease *Campylobacter* colonization during primary production might aid in reducing the number of human campylobacteriosis cases. Several plant-derived compounds have been reported to possess anti-*Campylobacter* properties *in vitro*, so their use could be a promising candidate to reduce *Campylobacter* colonization in broiler chickens. To test this hypothesis, first a selection (caffeic, gallic, protocatechuic and vanillic acid, epigallocatechin gallate, *trans*-cinnamaldehyde and thymol) of plant-derived antimicrobial compounds (PDAC) was screened for anti-*Campylobacter* activity by determining minimal inhibitory concentrations (MIC) and setting up time-kill curves for *C. jejuni* strain KC 40. These experiments revealed marked antibacterial activity especially for the cinnamon oil ingredient *trans*-cinnamaldehyde (CIN). For this reason, this compound was tested in a broiler chick seeder model in which it was added to the feed in coated form at an effective concentration of 0.3% from day-of-hatch for the entire 22-day duration of the experiment. At 14 days of age, one out of three birds was inoculated with *C. jejuni* strain KC 40 and served as seeders. CIN was not able to reduce cecal *Campylobacter* colonization in this model which was confirmed in a cecal loop experiment. Despite CIN concentrations much higher than the MIC, *C. jejuni* numbers were not reduced compared to those in non-treated ceca at two and 24 hours after injection. In conclusion, this study shows a marked discrepancy between *in vitro* and *in vivo* activity of *trans*-cinnamaldehyde against *C. jejuni* strain KC 40.

Key words: plant-derived antimicrobial compound; *trans*-cinnamaldehyde; *Campylobacter jejuni*; broiler chicken cecum

INTRODUCTION

Today, *Campylobacter* infections are the leading cause of bacterial gastro-enteritis in many developed countries (Moore *et al*., 2005; EFSA, 2010b), with *C. jejuni* being the main causative agent (EFSA, 2009). Poultry and poultry products are considered a major source for human infection (Vellinga & Van Loock, 2002; Friedman *et al*., 2004; Wingstrand *et al*., 2006). It is estimated that chickens as a reservoir would account for up to 80% of human campylobacteriosis cases (EFSA, 2010a). Despite many efforts directed to minimize contamination of poultry with *Campylobacter*, the overall number of reported poultry meat consumption-related human campylobacteriosis cases has not been reduced (EFSA, 2009, 2010b).

Due to the high prevalence of *Campylobacter* in poultry, chicken meat is often contaminated with this pathogen. *Campylobacter* colonization of the chicken intestinal tract plays an important role in carcass contamination during slaughter (Herman *et al*., 2003; Rosenquist *et al*., 2003). *Campylobacter* is found abundantly in the poultry cecum (Corry & Atabay, 2001), so that small amounts of cecal contents can already remarkably increase the amount of *Campylobacter* on eviscerated broiler carcasses (Berrang *et al*., 2004). Two independent studies estimated that by lowering *Campylobacter* numbers on chicken carcasses 100-fold, the number of campylobacteriosis cases in humans associated with consumption of chicken meals could be reduced by 84% (Messens *et al*., 2007) and 97% (Rosenquist *et al*., 2003), respectively. Thus, measures taken to reduce *Campylobacter* colonization in the intestinal tract of poultry during primary production have potential for reducing contamination of poultry products, and thus the incidence of human campylobacteriosis. Despite many efforts, however, no such intervention measure is available to date (Lin, 2009).

Combating cecal *Campylobacter* colonization in poultry by supplementing plant-derived antimicrobial compounds (PDAC) to the feed could be promising. Many plant-derived substances have the ability to kill a wide range of bacteria *in vitro*, including *Campylobacter* (Cowan, 1999; Friedman *et al*., 2002). To date, however, only a limited number of them have been tested for their ability to combat cecal *C. jejuni* colonization of broiler chicks.

The first aim of this study was to screen a selection of PDAC on their *in vitro* activity against the highly colonizing *C. jejuni* strain KC 40 (Van Deun *et al*., 2008a; Hermans *et al*., 2010). This selection was based on reported anti-*Campylobacter* activity *in vitro* previously: caffeic, gallic and vanillic acid (Gañan *et al*., 2009), epigallocatechin gallate (Nagayama *et al*., 2002), *trans*-cinnamaldehyde (Johny *et al*., 2008), protocatechuic acid (Yin & Chao, 2008) and

thymol (Anderson *et al*., 2009). The most active substances were tested further by setting up time-kill curves. The second objective was to evaluate the applicability of the most promising compound to reduce cecal *Campylobacter* colonization in broiler chickens.

MATERIALS AND METHODS

Experimental animals

Day-of-hatch Ross broiler chickens of both sexes from a local farm were raised in group until inoculation with *Campylobacter* and provided with feed and water *ad libitum*. Husbandry, euthanasia methods, experimental procedures and bio-safety precautions were approved by the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University, Belgium (EC numbers: 2009/114 and 2009/130). Chicks were examined for the presence of *Campylobacter* in mixed fecal samples. Samples were enriched in Nutrient Broth No.2 (NB2; Oxoid, Basingstoke, UK), supplemented with Modified Preston *Campylobacter* Selective Supplement (Oxoid) and *Campylobacter*-specific growth supplement (Oxoid). After 24 h incubation at 42°C under microaerobic conditions (5% O_2, 5% CO_2, 5% H_2, 85% N_2) enriched samples were plated on modified charcoal cefoperazone deoxycholate agar (mCCDA; Oxoid), supplemented with CCDA selective supplement (Oxoid) and *Campylobacter*-specific growth supplement, followed by microaerobic incubation at 42°C for 22 h. All mixed fecal samples proved to be *Campylobacter*-free prior to inoculation of chicks.

Bacterial strains and culture conditions

C. jejuni strain KC 40 from poultry origin was used in all experiments. Bacteria were routinely cultured for 17 h in NB2 with supplements at 42°C under microaerobic conditions (as above). *C. jejuni* bacteria were enumerated by preparing 10-fold dilutions in Hank's Balanced Salt Solution (HBSS; GIBCO-BRL, Invitrogen, Carlsbad, CA) and plating on mCCDA, followed by microaerobic incubation at 42°C for 22 h.

Minimal inhibitory concentration of plant-derived antimicrobial compounds against *Campylobacter jejuni*

The minimal inhibitory concentration (MIC) of plant-derived antimicrobial compounds (PDAC) against *C. jejuni* was determined three times in triplicate at pH 6.0 using HCl or NaOH to obtain the desired pH-values. Pure caffeic, vanillic, protocatechuic and gallic acid, as well as epigallocatechin gallate, *trans*-cinnamaldehyde and thymol (all products from Sigma, St.

Louis, MO) were first dissolved in absolute ethanol (VWR International, France) and then added in serial two-fold dilutions in 96-well plates in Mueller-Hinton (MH) broth (Oxoid) ranging from 15.6 to 2000 µ*M* (1000 µ*M* for caffeic acid). These tested concentrations were chosen based on preliminary studies (data not shown). Test dilutions were prepared in such a manner that final ethanol concentrations in none of the wells exceeded 0.75% (vol/vol), the ethanol concentration at which growth-inhibitory effects start to occur toward *C. jejuni* KC 40 (data not shown). The inoculum was prepared by adding a *C. jejuni* suspension, grown for 17 h in NB2 with supplements, to sterile phosphate buffered saline (PBS) until a turbidity equivalent to a 0.5 McFarland standard, and diluting 1:5 in MH broth. Five µL of this suspension was inoculated into 195 µL medium in each well so that the final inoculum contained approximately 5×10^5 cfu/mL. These suspensions were incubated for 24 h at 42°C in a microaerobic environment (as above) after which microbial growth was assessed macroscopically: the lowest concentration completely inhibiting visible growth was considered the MIC.

Time-kill curve experiments with selected PDAC

Time-kill curve experiments were conducted in MH broth without (growth control) or with the addition of epigallocatechin gallate (EGCG) or *trans*-cinnamaldehyde (CIN) at 125, 250, and 500 µ*M*. The solutions were brought to pH 6.0 using HCl or NaOH followed by inoculation with a *C. jejuni* KC 40 suspension grown for 17 h in NB2 with supplements, so the final bacterial concentration in these solutions was approximately 10^7 cfu/mL. After inoculation, samples were kept at 42°C in a microaerobic environment and bacterial counts were determined at 0, 1, 2, 4, 8, and 24 h post-inoculation (pi).

***In vivo* trial with supplementation of coated *trans*-cinnamaldehyde**

Day-of-hatch broiler chicks (n = 54) were randomly divided in six groups which were all housed separately. Birds of the first three groups were fed a standard feed and served as control birds. Chicks of the other three groups were fed the same standard feed, supplemented with 1% (wt/wt) coated cinnamaldehyde (cCIN; kindly provided by Koen Schwarzer, Nutri-Ad International, Kasterlee, Belgium), containing 30% (wt/wt) pure *trans*-cinnamaldehyde, so its final concentration in the feed was 0.3% (wt/wt), and 70% (wt/wt) coating material. Equal amounts of feed were provided for each group during treatment and care was taken that all animals had unlimited access to the feed. At the age of 15 days, three chicks of each group were orally inoculated with 1 ml of approximately 1.4×10^8 cfu of the highly colonizing *C.*

jejuni strain KC 40, allowing to cause infection in all animals of the same group ("seeder model") (Van Deun *et al*., 2008a). At 21 days of age all chicks were euthanized by injection of T61 (Intervet, Belgium) in the wing vain (500 µL/chick), and the ceca as well as their contents were collected for *C. jejuni* enumeration (see below).

Cecal loop model

A cecal loop model was used to examine the effect of CIN on cecal *Campylobacter* numbers in broilers. This model allows direct comparison of *C. jejuni* numbers in both ceca of the same animal. One cecum (positive control) is injected with a control solution, while in the other a test compound is injected (Van Deun *et al*., 2008b; Hermans *et al*., 2010). Day-of-hatch broiler chicks (n = 9) were raised in group. At the age of 13 days, chicks were divided in three groups (n = 3) and housed in separate isolation units. At the age of 14 days, all chicks of groups 1 and 2 were inoculated with *C. jejuni* KC 40 (approximately 10^8 cfu (group 1), respectively 2×10^7 cfu (group 2)), while chicks of group 3 were kept in a *Campylobacter*-free environment. Cecal loops were applied, according to Hermans *et al*. (2010), at the age of 18 days (groups 1 and 3), or 21 days (group 2). Test solutions were prepared by dissolving CIN in absolute ethanol and diluting in HBSS until the desired concentration. One of the ceca of each animal of group 1 and 2 was injected with 200 µL 100 m*M* CIN in HBSS, while the other was injected with 200 µL HBSS with 2.67% (vol/vol) absolute ethanol, and served as control. In the animals of group 3, one cecum was injected with 100 µL 40 m*M* CIN in HBSS, the other with 100 µL HBSS with 1.07% (vol/vol) ethanol. After this, both ceca were injected with 100 µL of a *C. jejuni* KC 40 suspension of approximately 6.5×10^4 cfu/mL in HBSS.
After injection, the cecum was repositioned in the abdominal cavity and the peritoneum, muscles and skin were sutured. After two h (group 2) or 24 h (groups 1 and 3) the chickens were euthanized by injection with T61 and the ceca with their contents were collected for *Campylobacter* enumeration (see below).

Cecal *Campylobacter jejuni* enumeration

Ceca and contents were cut into little fragments, weighed, and diluted 1:9 (wt/vol) in NB2 with supplements. After homogenization, a 10-fold dilution series was made in HBSS. Of each dilution, 100 µL was spread on mCCDA plates. After 22 h incubation at 42°C under microaerobic conditions, colonies were counted. For enrichment, diluted cecal samples in NB2 were incubated at 37°C under microaerobic conditions. After 24 h, samples were plated

on mCCDA and incubated at 42°C in a microaerobic environment. After 24 and 48 h plates were examined for the presence or absence of *C. jejuni*.

Statistical analysis

Data of the experiments were analyzed by SPSS 17.0 software for Windows. The significance level α was set at 0.05. *Campylobacter* counts were first transformed to $\log_{10}$ counts before statistical analysis. A one-way analysis of variance (ANOVA) was then carried out to compare the means of $\log_{10}$ transformed counts in broth (time-kill curves) or in chicken cecal contents of all groups (treated groups and control groups) of the seeder model. Significant differences were assessed by Dunnet (time-kill curves) or Bonferroni (seeder model) Post Hoc tests. *P*-values below 0.01 (time-kill curves) or 0.05 (seeder model) were considered significantly different. A paired-samples *t*-test was performed to compare the means of $\log_{10}$ transformed counts in chicken cecal contents of the treated group C and control group in the cecal loop experiment. *P*-values below 0.05 were considered significantly different.

RESULTS

MIC of all tested PDAC against *C. jejuni* KC 40

Table 2.1 shows the MIC values of the seven selected PDAC for poultry-derived *C. jejuni* strain KC 40 at pH 6.0. MIC value variation for each PDAC between the repeats was maximally one two-fold dilution. Epigallocatechine gallate (EGCG) and *trans*-cinnamaldehyde (CIN) had the lowest MIC value (125 μ*M*) compared to all other compounds (≥1000 μ*M*) for the tested strain.

Table 2.1 Overview of the minimal inhibitory concentration (MIC) of selected plant-derived antimicrobial compounds, tested in Mueller-Hinton (MH) broth at pH 6.0 against *Campylobacter jejuni* strain KC 40. MIC values were determined three times in triplicate

Compound	MIC value (μM)
caffeic acid	> 1000
vanillic acid	1000
gallic acid	2000
protocatechuic acid	> 2000
epigallocatechin gallate	125
trans-cinnamaldehyde	125
thymol	1000

Time-kill curves of selected PDAC against *C. jejuni* KC 40

Based on the MIC values obtained in the previous experiment, time-kill curves were conducted to analyze the bactericidal effect of EGCG and CIN toward *C. jejuni* KC 40 at pH 6.0, using concentrations of 125, 250 and 500 µ*M* over a 24-h period (**figure 2.1**). MH broth without acids and adjusted to pH 6.0 served as a growth control.

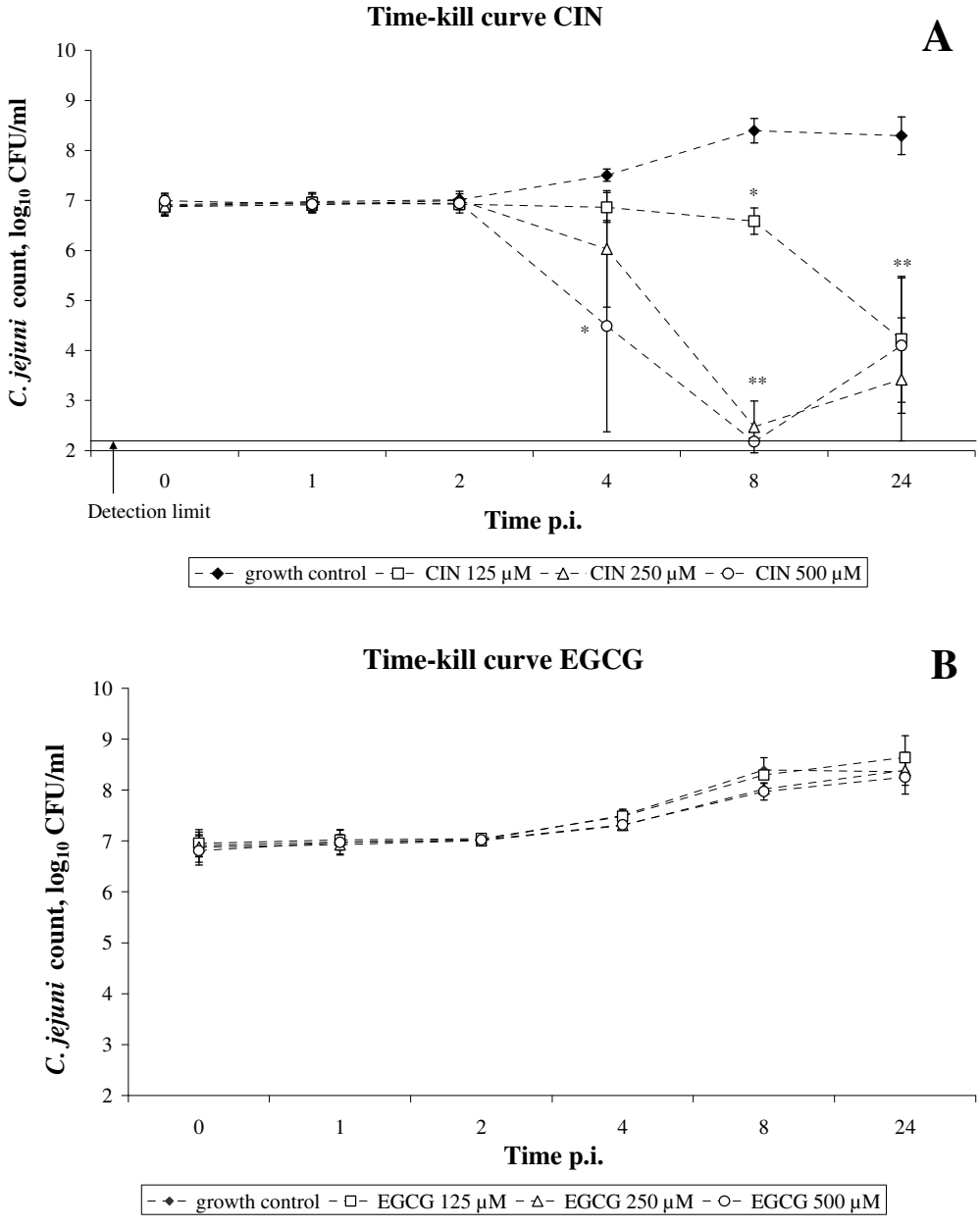

Figure 2.1 Time-kill curves of *trans*-cinnamaldehyde (CIN, panel A) and epigallocatechin gallate (EGCG, panel B) at pH 6.0 against *Campylobacter jejuni* strain KC 40. Bacterial counts are represented as $\log_{10}$ cfu/mL. ♦, growth control; □, CIN or EGCG at 125 µ*M*; ▲, CIN or EGCG at 250 µ*M*; ○, CIN or EGCG at 500 µ*M*. The experiment was performed in three independent trials and the mean results with standard deviation are shown. Significantly ($P < 0.01$) lowered bacterial counts compared with the control counts are denoted by an asterisk.

EGCG did not significantly ($P > 0.05$) reduce *C. jejuni* numbers (**figure 2.1, B**) at the concentrations tested. CIN was strongly bactericidal toward *C. jejuni* (**figure 2.1, A**) at all three concentrations tested. At 125 μ*M*, it was initially growth-inhibitory (after 8 h post-inoculation (pi)) and lead to a significant ($P = 0.01$) reduction of the initial bacterial counts of over two logs after 24 h. CIN concentrations of 250 μ*M* and 500 μ*M* were strongly bactericidal within 8 h pi as shown by a significant ($P \leq 0.01$) decrease of > 4 log. CIN at 500 μ*M* was already bactericidal within 4 h pi, as evidenced by significantly ($P \leq 0.05$) reduced (~ 3 log) bacterial counts compared with the control counts. Also after 24 h, both concentrations of CIN significantly ($P \leq 0.01$) reduced *C. jejuni* counts, which re-established to a level ca. three logs below the initial inoculum counts.

In-feed cCIN does not reduce cecal colonization with *C. jejuni* in broiler chicks

The three groups of 21-day-old broilers in the seeder model receiving feed supplemented with 0.3% (wt/wt) cCIN did not have significantly ($P > 0.05$) lower cecal *Campylobacter* counts, compared with the three groups receiving unsupplemented feed (**figure 2.2**). Feed uptake (data not shown) was similar for all groups during the entire duration of the experiment.

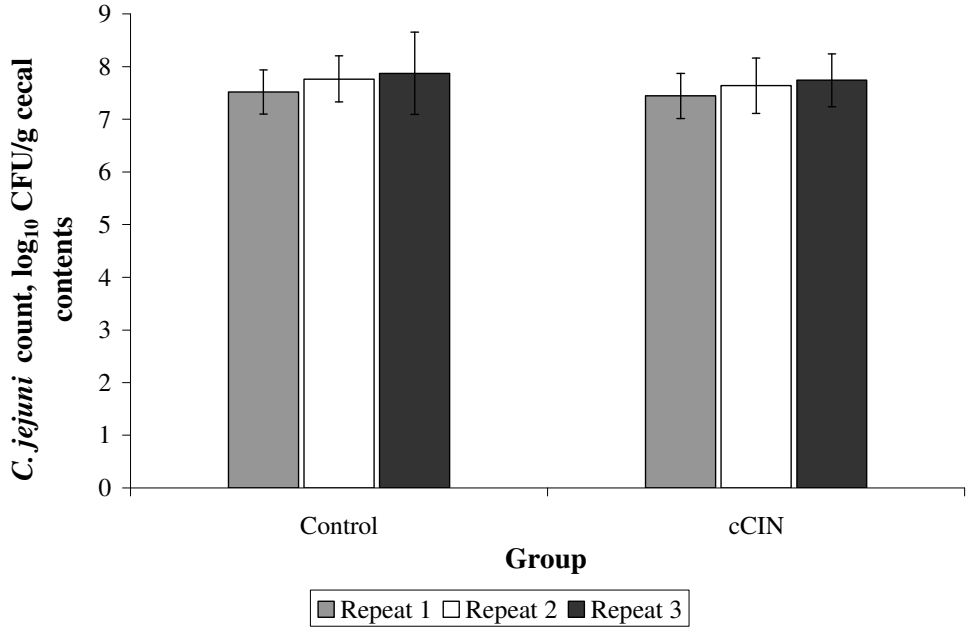

Figure 2.2 Average cecal *Campylobacter jejuni* counts in 21-day-old broilers (n = 9 chickens/group) fed unsupplemented feed or coated *trans*-cinnamaldehyde (cCIN) at 1% (wt/wt) for the entire duration of the experiment. Three chicks of each group were orally inoculated with approximately 1.4×10^8 cfu of *C. jejuni* strain KC 40 at 14 days of age and served as seeders. All birds were euthanized at 21 days of age. Values are represented as the mean of $\log_{10}$ cfu/g cecal contents ± standard deviation (n = 9). The experiment was performed in triplicate.

CIN is not able to reduce *C. jejuni* numbers in the cecal loop model

The cecal content of 18- to 21-day-old broilers was estimated to be 1.0–2.0 mL. By injecting 100 µL of a 40 m*M* (as well as 100 µL *Campylobacter* suspension) or 200 µL of a 100 m*M* CIN solution, final concentrations in these ceca ranged from 1.8–3.3 m*M* or 9.1–17 m*M*. This was largely sufficient to cause a growth-inhibitory, bactericidal effect toward *C. jejuni* under *in vitro* conditions (see above). Final ethanol concentrations in all ceca were < 0.5% (vol/vol), thus beneath the growth-inhibitory level (see above). Despite these CIN concentrations, neither at 2 h nor at 24 h after injection mean *Campylobacter* levels in the cecal loops of broilers was reduced with our preset $\log_{10}$ 2, compared with the control cecum (**figure 2.3**).

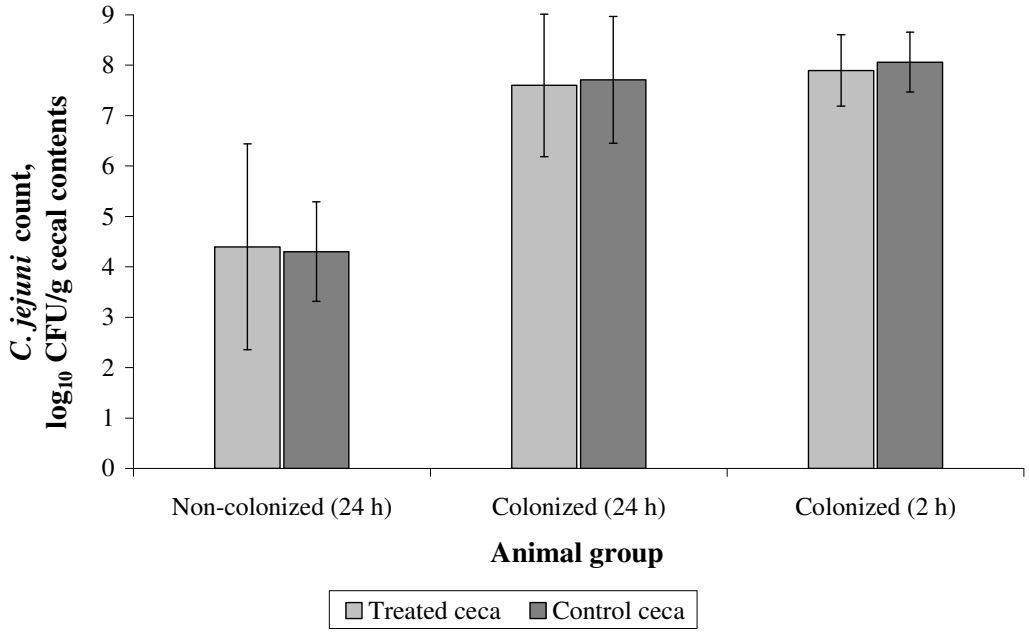

Figure 2.3 *Campylobacter jejuni* counts in cecal loops of 19-day-old (n = 2 chickens/treatment) and 21-day-old (n = 3) broilers. In the left animal group, one cecum of a *Campylobacter*-free chick was injected with 100 µL of 40 m*M trans*-cinnamaldehyde (CIN), the other with 100 µL Hanks' Balanced Salt Solution (HBSS). Both ceca were injected with 100 µL of a 6.5×10^4 cfu/mL *C. jejuni* suspension and *C. jejuni* counts were determined 24 h later. In the middle and right animal group, one cecum of a *C. jejuni*-infected chick was injected with 200 µL 100 m*M* CIN, the other with 200 µL HBSS and *C. jejuni* counts were determined 24 h (middle) or 2 h (right) later. Values are represented as $\log_{10}$ cfu/g cecal contents.

DISCUSSION

The first objective of this study was to screen for the plant-derived antimicrobial compound (PDAC) with the highest *in vitro* activity against the highly colonizing *C. jejuni* strain KC 40

out of a selection of substances already reported to possess marked anti-*Campylobacter* activity *in vitro*. Minimal inhibitory concentrations (MIC) of seven PDAC toward *C. jejuni* were determined at pH 6. This pH approaches the mean cecal pH of broilers during their life-cycle (van der Wielen *et al*., 2000) and compensates for any pH-dependent effect. This approach revealed two compounds in particular to be very active against *C. jejuni* KC 40 with a MIC of 125 μ*M*: the green tea polyphenol epigallocatechin gallate (EGCG) and the cinnamon oil ingredient *trans*-cinnamaldehyde (CIN). Time-kill curve experiments revealed bactericidal properties toward *C. jejuni* at pH 6 only for CIN at the tested concentrations: at 125 μ*M* already, this compound was able to reduce bacterial counts after 24 h. However, bacterial counts tended to re-establish after an initial drop. Nevertheless, a marked bactericidal, dose-dependent activity of CIN toward *C. jejuni in vitro* was observed, as reported previously (Friedman *et al*., 2002; Johny *et al*., 2008; Ravishankar *et al*., 2008). Friedman *et al*. (2002) previously screened a more extensive selection of 23 essential oils and 96 oil compounds and found *trans*-cinnamaldehyde to be one of the most active substances toward *C. jejuni in vitro*. Hence, CIN seems a promising candidate to use as an in-feed PDAC for reducing cecal *C. jejuni* colonization of broiler chickens.

To our knowledge, the ability of CIN to reduce cecal *Campylobacter* colonization in broilers has not yet been examined. Therefore, our second objective was to analyze in the broiler cecum the anti-*Campylobacter* activity of CIN, classified as generally recognized as safe (GRAS) by the Food and Drug Administration (FDA). Coating a compound on micro-beads is indispensable in a classic *in vivo* experiment where one administers the compound through the feed to allow it to reach the small intestine and the ceca (Van Immerseel *et al*., 2004), the latter representing the predominant site for *Campylobacter* colonization (Beery *et al*., 1988). Administration of micro-encapsulated CIN (cCIN) to the feed at 1% (wt/wt) was not shown to reduce feed uptake and was therefore chosen for the *in vivo* trial. Coating material consisted of fatty acids, mainly palmitic acid. When reaching the gut, CIN is released from the coating material by hydrolytic activity from the gut lipases. cCIN-supplemented feed, containing 0.3% (wt/wt) pure *trans*-cinnamaldehyde, did not reduce cecal *Campylobacter* colonization in 21-day-old chicks in our seeder model, where one on three birds was infected at 14 days of age. It is known that cinnamaldehyde is degraded very quickly in the digestive tract of piglets (Michiels *et al*., 2008). This might be extrapolated to the broiler gastro-intestinal (GI) tract and point to the utmost importance of developing a suitable coating material if one aims to obtain bactericidal CIN concentrations in more downstream areas such as the broiler cecum. It might thus be possible that the coating material used in this study was not capable of

bypassing the premature degradation of CIN along the chicken GI tract leading to a lack of effect in decreasing cecal *Campylobacter* levels in treated versus control groups. To examine whether CIN is able to target *C. jejuni* in the broiler cecum, a cecal loop model, already successfully applied in previous studies (Van Deun *et al*., 2008b; Hermans *et al*., 2010), was used. Here, one directly injects the test compound into the ceca, making it unnecessary to coat the compound. Twenty-four h after injection, CIN was not able to reduce *Campylobacter* numbers in the cecal loops of broilers experimentally infected with *C. jejuni* strain KC 40 at 14 days of age or at the time of injection, despite concentrations much higher then the MIC. To examine whether the lack of effect after 24 h was due to a re-establishment to original *C. jejuni* counts after an initial drop, a group of animals was euthanized 2 h after CIN injection. Also here, however, no reduction in cecal *Campylobacter* counts was observed.

Combined, these results suggest that *C. jejuni* is occupying a protected niche in the ceca of broilers rendering them less susceptible to the bactericidal effects of CIN observed *in vitro*. Also for capric acid we already showed that despite its marked anti-*Campylobacter* activity *in vitro*, no reduction in *Campylobacter* counts in the broiler cecum could be obtained, both in a cecal loop model as well as in a classic *in vivo* experiment (Hermans *et al*., 2010). These observations put the possible use of in-feed PDAC to combat cecal *Campylobacter* colonization in poultry in question. To make clear, however, if the observed feature for capric acid and CIN can be extrapolated to all PDAC, there is need for an *in vitro* fermentation model mimicking the broiler's cecal environment. Moreover, recent studies reported observations that are not in line with our hypothesis. It was shown that the probability of the bacterium to reach and colonize the broiler cecum can be reduced by supplementing medium-chain fatty acids to the broiler feed (Solis de los Santos *et al*., 2008a; van Gerwe *et al*., 2009) or even completely prevented with a combination of formic acid and sorbate (Skånseng *et al*., 2010). Additionally, caprylic acid was shown to decrease cecal *Campylobacter* numbers by multiple logs both in 15-day-old (Solis de los Santos *et al*., 2008b) and market-aged (Solis de los Santos *et al*., 2009) broilers. It is clear that further research will have to elucidate the potential contribution of in-feed PDAC to effectively combat cecal *Campylobacter* colonization in chicks.

In conclusion, we here show a marked discrepancy between *in vitro* and *in vivo* activity of the cinnamon-oil ingredient *trans*-cinnamaldehyde: neither in a classic *in vivo* trial nor in the cecal loop experiment CIN was able to reduce cecal numbers of *C. jejuni* strain KC 40, despite marked *in vitro* activity.

Acknowledgements

This work was financed by a grant of the Federal Public Service of Health, Food Chain Safety and Environment (FOD, Brussels, Belgium): project RT08/8-CAMPOUL. Coated *trans*-cinnamaldehyde was kindly provided by Koen Schwarzer (Nutri-Ad International, Kasterlee, Belgium).

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CHAPTER 3: EXPERIMENTAL STUDIES

3.3 PASSIVE IMMUNIZATION TO REDUCE *CAMPYLOBACTER JEJUNI* COLONIZATION AND TRANSMISSION IN BROILER CHICKENS

SUMMARY

Campylobacter jejuni is the most common cause of bacterial-mediated diarrheal disease worldwide. Poultry products are considered the most important source of *C. jejuni* infections in humans but to date no effective strategy exists to eradicate this zoonotic pathogen from poultry production. Here, the potential use of passive immunization to reduce *Campylobacter* colonization in broiler chicks was examined. For this purpose, laying hens were immunized with either a whole cell lysate or the hydrophobic protein fraction of *C. jejuni* and their eggs were collected. *In vitro* tests validated the induction of specific ImmunoglobulinY (IgY) against *C. jejuni* in the immunized hens egg yolks, in particular. In seeder experiments, preventive administration of hyperimmune egg yolk significantly ($P < 0.01$) reduced bacterial counts of seeder animals three days after oral inoculation with approximately 10^4 cfu *C. jejuni*, compared with control birds. Moreover, transmission to non-seeder birds was dramatically reduced (hydrophobic protein fraction) or even completely prevented (whole cell lysate). Purified IgY did not affect motility but promoted bacterial binding to intestinal mucus, suggesting enhanced mucosal clearance *in vivo*. Western Blot analysis followed by electrospray ionization mass spectrometry of in-gel digested, 2-dimensional separated proteins identified most of the immunodominant antigens of *C. jejuni*, which could be classified as involved in a variety of cell functions, including chemotaxis and adhesion. Some of these (AtpA, EF-Tu, GroEL and CtpA) are highly conserved proteins and could be promising targets for the development of subunit vaccines.

Key words: *Campylobacter jejuni*; chicken; passive immunization; hyperimmune egg yolk

INTRODUCTION

Today, *Campylobacter jejuni* is the most common cause of bacterial-mediated diarrheal disease in humans, worldwide (EFSA, 2011a). Despite many efforts aimed at minimizing *Campylobacter* contamination of poultry, no effective, reliable intervention measures exist to reduce bacterial numbers in the broiler gut (Hermans *et al*., 2011a). In general, broiler chickens become colonized only at an age of two weeks (van Gerwe *et al*., 2009). Transportation of immunoglobulin (Ig) Y, the major Ig class in chickens, from the hen to the embryo via egg yolk is believed to play a key role in the protection of young chicks with an immature immune system against *Campylobacter* colonization during two to three weeks post-hatch (Chalghoumi *et al*., 2009a; Cawthraw & Newell, 2010). From two weeks onward, the concentration of maternally derived anti-*Campylobacter* IgY drops significantly, which coincides with an increased colonization susceptibility of the broiler chicks. Passive immunization of chicks may be prolonged by feeding broilers with high levels of anti-*Campylobacter* antibodies recovered from immunized hens (Chalghoumi *et al*., 2009a). Following immunization, specific IgY is induced and transferred from the serum to the egg yolk (Chalghoumi *et al*., 2009a), where it is accumulated at high levels (Dias da Silva & Tambourgi, 2010). Indeed, pre-incubating *C. jejuni* with IgY from immunized hens has previously been shown to reduce fecal *C. jejuni* counts in broilers experimentally inoculated with this mixture (Tsubokura *et al*., 1997). In this study, also the effect of IgY on colonization in already-colonized animals was assessed. Results indicated that IgY induced only a limited therapeutic efficacy. After an initial drop, fecal *C. jejuni* numbers regained their original counts when treatment was stopped. Nevertheless, these observations indicate that IgY preparations from egg yolks could be a promising candidate to reduce *C. jejuni* colonization in broilers, but need to be optimized because no studies regarding this topic were reported since. Also, under farming conditions pre-incubation of the colonizing *C. jejuni* strains with IgY is not feasible. Moreover, in the study by Tsubokura *et al*. (1997) faecal bacterial counts were determined, which may not perfectly correlate to determining cecal counts, which is more sensitive (Woldemariam *et al*., 2008). In the past two decades, some promising observations have been reported concerning the use of chicken egg yolk immunoglobulins for the prevention against other bacterial and viral pathogens as well, including amongst others streptococci (Smith *et al*., 2001), *Helicobacter pylori* (Shimamoto *et al*., 2002), human rotavirus, enterotoxigenic *Escherichia coli*, *Yersinia ruckeri* and *Salmonella* species (Mine &

Kovacs-Nolan, 2002; Chalghoumi *et al*., 2009a), using animal models or tested in humans. In these studies, protection was primarily observed after infection with the homologous strain. Passive immunization using yolk IgY thus seems a promising strategy to control *C. jejuni* colonization in broiler flocks. For this purpose, the first aim of this study was to examine whether artificial passive immunization by feeding experimentally inoculated broilers with hyperimmune eggs could reduce or prevent cecal *C. jejuni* colonization in broiler chicks. The second objective was to identify the *C. jejuni* antigens which were responsible for the induction of *C. jejuni*-specific IgY.

MATERIALS AND METHODS

Experimental animals

Commercial brown Leghorn laying hens and day-of-hatch Ross broiler chickens of both sexes from a local farm were raised in group until treatment. Birds were provided with a commercial feed and water *ad libitum*. Husbandry, euthanasia methods, experimental procedures and bio-safety precautions were approved by the Ethical Committee (EC) of the Faculty of Veterinary Medicine, Ghent University, Ghent, Belgium (EC numbers: 2010/174 and 2011/029). Chicks were examined for the presence of *Campylobacter* in mixed fecal samples and proved to be free of *Campylobacter*.

Bacterial strains and culture conditions

Campylobacter jejuni strain KC 40 from poultry origin was used for all experiments. This strain colonizes chickens to a high level (Van Deun *et al.,* 2008; Hermans *et al*., 2010; Hermans *et al*., 2011c). Bacteria were routinely cultured in Nutrient Broth No.2 (NB2, CM0067; Oxoid Ltd., Basingstoke, Hampshire, UK) supplemented with Modified Preston *Campylobacter*-selective supplement (SR0204E; Oxoid) and *Campylobacter*-specific growth supplement (SR0232E; Oxoid), at 42°C under microaerobic conditions (5% O_2, 5% CO_2, 5% H_2, 85% N_2). *C. jejuni* bacteria were enumerated by preparing 10-fold dilutions in Hank's Balanced Salt Solution (HBSS; GIBCO-BRL, Invitrogen, Carlsbad, CA) and plating on modified charcoal cefoperazone deoxycholate agar (mCCDA, CM0739; Oxoid) supplemented with CCDA selective supplement (SR0155E; Oxoid) and *Campylobacter*-specific growth supplement (SR0232E; Oxoid), followed by microaerobic incubation at 42°C for 22 h.

Extraction of *C. jejuni* hydrophilic and hydrophobic proteins

To prepare the immunizing agent, cells from a *C. jejuni* culture were collected by centrifugation (5000 × g for 30 min at 4°C), washed in HBSS and sonicated on ice for 30 seconds in extraction buffer 1 (EB1; 40 m*M* Tris, supplemented with tributylphosphine solution (Sigma), protease and phosphatase inhibitors, DNase and RNase) using a tip sonicator (Qsonica, Newtown, VS). After each sonication step, 12 in total, the sample was left on ice for 30 seconds. After centrifugation at 16,000 × g for 10 min at 4°C the supernatant (containing the hydrophilic proteins) was collected and the pellet resuspended in extraction buffer 2 (EB2; 40 m*M* Tris, supplemented with 5 *M* urea, 2 *M* thiourea, 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 0.2% carrier ampholytes, 100 m*M* dithiothreitol and the respective inhibitors). After sonication and centrifugation of this suspension at 5,000 × g for 3 min to remove cell debris and unlysed cells, the supernatant (containing the hydrophobic proteins) was either pooled with the first supernatant (to obtain the whole cell protein lysate) or stored (4°C) separately.

Immunization of laying hens

Commercial Brown Leghorn chickens were randomly assigned to vaccination groups at the age of 19 weeks. Chicks were immunized with either the whole *C. jejuni* KC 40 cell lysate (EB1 + EB2) or 25 µg protein of the hydrophobic protein fraction only (EB2), by intramuscular injection of 250 µL of a 1:1 mixture with Freunds' Complete Adjuvant at four different sites of the pectoral muscle. Chicks of the control group were immunized with a mixture of HBSS and Freunds' Complete Adjuvans (FCA). Three booster immunizations using Freunds' Incomplete Adjuvans (FIA) were given in a two-weekly time interval. Starting from one week after the second boost, eggs of the animals were collected and stored at 4°C.

Determination of egg yolk and white IgY and IgA titers

At weekly time intervals, starting from day 1, eggs were collected from both groups. The egg yolk and white were separated and pooled per group, diluted 1:5 (wt/vol) in distilled water and mixed thoroughly. After overnight incubation at 4°C the supernatant, containing the water-soluble fraction of the egg yolk and white, respectively, was collected for immunoglobulin quantification using enzyme-linked immunosorbent assay (ELISA). Wells of a 96 well Nunc-MaxiSorp microtiter plate were coated with 50 µL of a suspension of *C. jejuni* KC 40 cells (2×10^7 cfu/mL) in coating buffer (2.16 g $NA_2CO_3.10H_2O$ + 1.935 g $NaHCO_3$ in 500 mL H_2O) that were either heat-killed or processed to antigenic fractions by sonication.

Plates were incubated overnight at 4°C after which the wells were washed three times with HBSS followed by a final wash step using wash buffer (WB; PBS + 0.1% Tween 20). Next, the wells were incubated with 100 µL blocking buffer (BB; WB + 1% bovine serum albumin) at room temperature (RT) for 1 h to reduce non-specific binding of antibodies. Subsequently, 100 µL of doubling dilutions (in BB) of the supernatant was added to the wells in triplicate. The plates were incubated at RT for 90 minutes. After incubation the wells were washed three times using HBSS and once using WB and 100 µL of 1/10000 (in WB) of horseradish peroxidase (HRP)-labeled anti-chicken IgY (Sigma) or 1/20000 HRP-labeled goat anti-chicken IgA (Bio-Connect, The Netherlands) was added to each well. After incubation and washing as described above, 50 µL of 3,3',5,5'-tetramethylbenzidine (TMB; Sigma) substrate was added. After 10 minutes the reaction was blocked by adding 50 µL 0.5 *M* H_2SO_4 to the wells. The absorbance was then measured at 450 nm using an automated spectrophotometer. Antibody titres from egg yolks/whites of immunized hens were reported as the highest dilution where the optical density (OD) was greater than the OD + three standard deviations of wells containing yolk/white originating from birds that only received adjuvans.

Purification of IgY fraction from egg yolks

IgY was purified from the hen eggs by the method of Bird & Thorpe (2002). Briefly, egg shells were broken and the yolk was separated from the white. Fifty mL of pooled yolk was placed on a filter paper in which a small whole was cut and pierced with a needle to loosen the membrane. Yolks were collected and diluted 1:5 (wt/vol) in Tris-buffered saline (TBS): 0.14 *M* NaCl in 10 m*M* Tris-HCl at pH 7.4. The solution was centrifuged at 2800 × g for 20 min at RT and the supernatant was collected. Subsequently, 120 µL dextran sulphate solution (10% in TBS) was added per 1 mL of supernatant and the solution was mixed well and incubated at RT for 30 min. Then, 50 µL of 1 *M* $CaCl_2$ was added per mL solution, mixed well and incubated for a further 30 min at RT after which the solution was centrifuged at 2800 × g for 30 min. The supernatant was collected and 20% (wt/vol) sodium sulphate was added while stirring. After complete dissolution, the solution was left standing for 30 min and centrifuged at 2800 × g for 20 min. The precipitate was redissolved in 10 mL TBS and centrifuged at 2800 × g for 20 min. The supernatant was collected and 8 mL 36% (w/v) sodium sulphate solution was added. The solution was left standing for 30 min at RT and centrifuged at 2800 × g for 20 min afterwards. The pellet was redissolved in 5 mL TBS. This solution was dialyzed against TBS and the purified immunoglobulin was filter sterilized.

Collection of broiler chicken intestinal mucus

Commercial and *Campylobacter*-free 14-d-old broiler chicks were euthanized and the small intestine was collected and gently rinsed with PBS to remove fecal material. The mucus was scraped from the mucosa with a glass slide covered in parafilm, diluted 1:3 with N-2-hydroxyethylpiperazine-N9-2-ethanesulfonic acid (HEPES, 25 m*M*, pH 7.4) and vortexed. The solution was centrifuged three times at 2000 × g for 15 min at 4°C. The supernatant containing the crude mucus was centrifuged two times more at 12,000 rpm for 15 min at 4°C and stored at -80°C. Protein content was determined using a Biorad protein assay kit (Biorad, Nazareth, Belgium).

Total protein content

Total protein content in mucus, IgY and *C. jejuni* lysate samples was determined using a Coomassie (Bradford) Protein Assay Kit according to the manufacturer's instructions.

Mucus adhesion test

To examine the effect of IgY on the adherence of *C. jejuni* to intestinal mucus, bacteria were first incubated for 30 min at 42°C in HBSS only or supplemented with purified IgY from *C. jejuni*-immunized or HBSS-immunized hens. Mucus was diluted to a final concentration of 250 µg protein/mL in coating buffer. One hundred µL was immobilized per well of a maxisorb microtiter well plate and incubated overnight at 4°C. The wells were washed three times with HBSS and saturated with 1% (wt/vol) BSA in HBSS for 1 h at RT. After washing the wells two times with HBSS, 100 µL of the *C. jejuni*-IgY mixture was transferred to the wells. After incubation for 1 h at 42°C the wells were washed 15 times to remove unbound bacteria. Wells were thereafter treated with 200 µL 0.5% (vol/vol) Triton X-100 to desorb adherent bacteria and incubated for 30 min at RT while shaking. Next, 300 µL HBSS was added to each well and 10-fold dilutions of the wells were titrated on mCCDA plates.

Motility assay

To assess the influence of IgY on *C. jejuni* KC 40 motility, bacteria were pre-incubated with purified IgY and 20 µL was pipetted onto semi-solid Mueller-Hinton (MH) agar. After 24 h incubation at 42°C under microaerobic conditions, growth was assessed.

Effect of in-feed hyperimmune egg yolk on transmission of and cecal colonization with *C. jejuni* in two-week-old broilers

In trial 1, day-of-hatch broiler chicks (n = 22) were raised in group. At 6 days of age the chicks were randomly assigned to 2 groups (n = 11/group) and housed in separate isolating chambers. Animals of group 1 were provided with feed containing 5% (wt/wt) egg yolk (mixed manually through the feed) from hens immunized with HBSS/adjuvans, while birds of group 2 were fed 5% (wt/wt) egg yolk from hens immunized with a whole cell *C. jejuni* lysate/adjuvans mixture. Egg yolks were added to the feed for the remainder of the experiment. Equal amounts of feed and drinking water were provided for each group during treatment and care was taken that all animals had unlimited access to the feed and water. At the age of 10 days, three chicks of both groups were orally inoculated with approximately 8×10^3 cfu of *C. jejuni* strain KC 40. At 13 days of age all animals were euthanized (as described above) and the ceca as well as their contents were collected for *C. jejuni* enumeration (see below).

Trial 2 was performed analoguously. Day-of-hatch broiler chicks (n = 54) were raised in group. At 6 days of age the chicks were randomly assigned to 6 groups (n = 9/group) and housed in separate isolating chambers. Animals of groups 1, 2 and 3 were provided with feed containing 5% (wt/wt) egg yolk from hens immunized with HBSS/adjuvans, while birds of groups 4, 5 and 6 were fed 5% (wt/wt) egg yolk from hens immunized with the hydrophobic protein fraction of *C. jejuni* mixed with adjuvans. At the age of 10 days, three chicks of each group were orally inoculated with approximately 3×10^4 cfu of *C. jejuni* strain KC 40.

Cecal *Campylobacter jejuni* enumeration

Ceca and contents were cut into little fragments, weighed, and diluted 1:9 (wt/vol) in NB2 with supplements. After homogenization, a 10-fold dilution series was made in HBSS. Of each dilution, 100 µL was spread on mCCDA plates. After 22 h incubation at 42°C under microaerobic conditions, colonies were counted. For enrichment, diluted cecal samples in NB2 were incubated at 37°C under microaerobic conditions. After 24 h, samples were plated on mCCDA and incubated at 42°C in a microaerobic environment. After 24 and 48 h plates were examined for the presence or absence of *C. jejuni*.

Western blot analysis

To identify the immunodominant antigens of *C. jejuni* recognized by yolk IgY of immunized hens, the whole *C. jejuni* lysate was separated by iso-electric focussing (pH 3-10), followed

by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a 4% stacking gel and 10% separating gel at 150V for 30 min and 200V for 60 min. The separated proteins were transferred electrophoretically (50V) onto nitrocellulose membranes (Bio-Rad) for 3 h. After washing with milliQ water, the membrane blots were blocked in 5% (wt/vol) milk in PBS for 1 h at RT. Subsequently, the blots were incubated overnight with purified egg yolk IgY, diluted to 1:5000 (vol/vol) in 5% (wt/vol) milk. After washing with 0.05% (vol/vol) Tween-20, the blots were incubated with horseradish peroxidase-conjugated goat-anti-chicken immunoglobulin G at a dilution of 1:50,000 in 5% (wt/vol) milk for 1 h. The blots were washed and proteins were immunodetected by enhanced chemiluminiscence, using Supersignal West Dura Extended Duration Substrate (Pierce), and scanned and digitized using the Versa Doc imaging system. Blotting experiments were performed in duplo. To identify the immunodominant antigens during immunization of layer hens that may play a critical role in protective passive immunity in broiler chicks, the egg yolk IgY responses of HBSS-immunized and *C. jejuni*-immunized layers were compared by immunoblotting them with 2-dimensially separated antigens. *C. jejuni* antigens reacting with hyperimmune egg yolk IgY were digested (see below) and sequenced (see below).

In-gel digestion

Spots of interest were cut into small gel pieces and washed twice in 25 m*M* ammoniumbicarbonate (ABC) with 50% acetonitril (ACN) for 10 min while shaking. Next, proteins were reduced in 10 m*M* dithiothreitol (DDT) with 25 m*M* ABC at 56°C, brought to RT and incubated further for 20 min. Alkylation was performed in 100 m*M* iodacetamide with 25 m*M* ABC for 45 min at RT and washed as describe above. Next, gel pieces were dehydrated in 100% ACN until they turned opaque and treated with trypsin (10 ng/µL) for 30 min on ice. After overnight incubation at 37°C, peptides were extracted in 50% ACN for 30 min. The samples were centrifuged at 15,000 × g and the supernatant was collected. The pellet was resuspended in 100% ACN, incubated for 30 min and centrifuged at 15,000 × g. The supernatant was collected and pooled with the first supernatant. Samples were dried and stored at -20°C.

Mass spectrometric analysis

Liquid chromatography

Prior to mass spectrometry the isolated peptides were separated on a U3000 nano-HPLC device (Dionex). Samples were first desalted on a pre-column (Acclaim PepMap 100 C18, 0.3

mm internal diameter (i.d.) × 5 mm, 5 µm particle size) at 20 µL/min with 0.1% formic acid for 10 min and subsequently separated on an analytical column (Pepmap C18 column 15 cm, particle size x µm, x mm internal diameter by 150 mm, Dionex). The gradient consisted of buffer A (0.1% formic acid (FA)) and buffer B (80% acetonitrile in 0.1% FA). The flow rate was 300 nL/min and the gradient elution was performed as followed: 30 min at 100% A, followed by a linear gradient of 4% B to 100% B over 70 min. Subsequently, an equilibration was performed for 10 min at 100% A. The column temperature was set at 60°C.

Mass spectrometric analysis

Identification of the peptides was performed on an electrospray ionization quadripole time of flight (ESI-QUAD-TOF) spectrometer (Ultima, Waters). Peptides were acquired at the range of 450 to 1650 m/z in MS. The seven most abundant multiple charges ions with a minimum intensity of 60 counts per second were subjected to MS/MS (between 50 and 2300 m/z). m/z ratios selected for MS/MS were excluded for 150 seconds.

Data analysis was performed against the *Campylobacter* protein databank from NCBI using the in-house search engine Mascot Daemon (2.3, Matrix Science, London, UK). An error tolerant search was performed with carbamidomethyl (C) as fixed modification. Deamidated (NQ) and oxidation (M) were set as variable modifications. Peptide mass tolerance and fragment mass tolerance were set at 0.35 Da and 0.6 Da, respectively. Maximum two miss cleavages were allowed. Proteins were only considered to be positively matched if the significance was below 0.05 ($p \leq 0.05$) and at least one peptide passing the required bold red criteria from Mascot Daemon, indicating that at least one peptide had rank 1 with a significance below 0.05.

Uncentroiding for MS processing was performed at a peak half width of 0.2. The fitted model for MS peak picking was isotope distribution. The minimum signal to noise was set at 2 for MS as well as MS/MS.

Statistical analysis

Data of the *in vivo* trials were analyzed by SPSS 17.0 software for Windows. The significance level α was set at 0.05. *Campylobacter* counts were first transformed to $\log_{10}$ counts before statistical analysis. A one-way analysis of variance (ANOVA) was then carried out to compare the means of $\log_{10}$ transformed counts in chicken cecal contents of all groups (treated groups and control groups). Significant differences were assessed by Bonferroni Post Hoc tests. *P*-values below 0.05 were considered significantly different.

RESULTS

Determination of antibody titers from egg

In our study, *C. jejuni*-specific IgY was dramatically induced in egg yolk of hens immunized with a *C. jejuni* lysate, resulting in titers of up to 1:16,000 as determined by ELISA (results not shown). In contrast, *C. jejuni*-specific IgY in egg white and *C. jejuni*-specific IgA in egg white and yolk were not significantly induced after immunization and were present at only marginal concentrations in both groups.

In vitro anti-*Campylobacter* properties of egg yolk IgY

Pretreatment with purified IgY from yolks of *C. jejuni*-immunized hens significantly ($p < 0.05$) promoted bacterial binding of the homologous *C. jejuni* strain KC 40 to chicken intestinal mucus compared to IgY from HBSS-immunized (control) animals (**figure 3.1**). In contrast, *C. jejuni*-specific IgY did not affect *C. jejuni* KC 40 motility (data not shown).

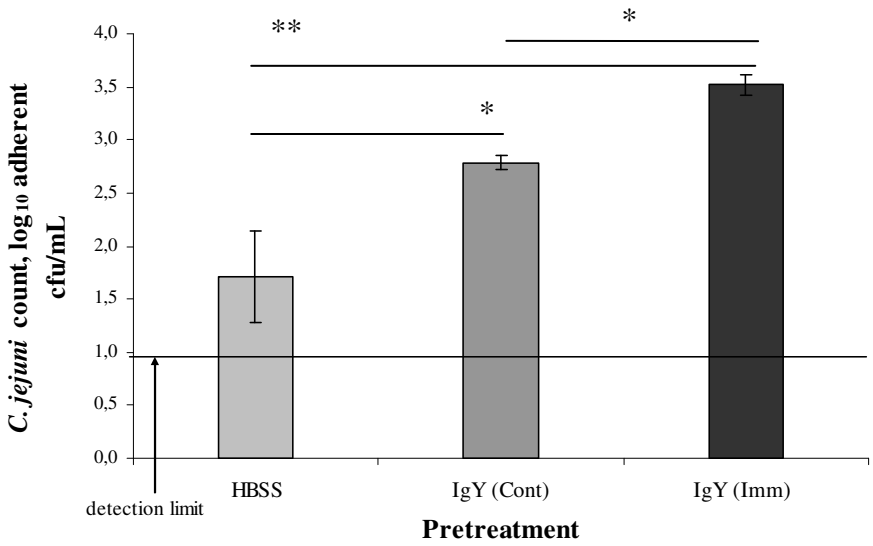

Figure 3.1 Total adherent *Campylobacter jejuni* bacteria to chicken intestinal mucus after pre-incubation with HBSS (control,) or IgY derived from *C. jejuni*-immunized ( ) or HBSS-immunized hens ( ). Values are represented as log cfu adherent bacteria/well. Statistical differences are denoted with one ($p < 0.05$) or two ($p < 0.01$) asterices.

In vivo anti-*Campylobacter* properties of egg yolk IgY

In *in vivo* trial 1, cecal *C. jejuni* numbers (**figure 3.2**) of inoculated broilers receiving hyper-immune egg yolks from whole cell *C. jejuni* lysate-immunized layers were reduced by > 5 logs ($p = 0.003$) compared to control broilers (3.3 ± 1.2 vs. 8.4 ± 0.6 ($\log_{10}$ cfu/g cecal contents)). Moreover, transmission to non-seeder chicks was completely prevented, in

contrast to the control group where non-seeder birds were all colonized at a similar (p = 0.143) degree to seeder chicks (7.3 ± 1.1 vs. 8.4 ± 0.6). Together, this lead to a dramatic reduction ($p < 0.001$) in the overall cecal *C. jejuni* count by $\log_{10}$ 6.7 for birds receiving hyperimmune yolk (0.9 ± 1.6 $\log_{10}$ cfu/g cecal contents) compared to birds receiving control yolks (7.6 ± 1.1 $\log_{10}$ cfu/g cecal contents).

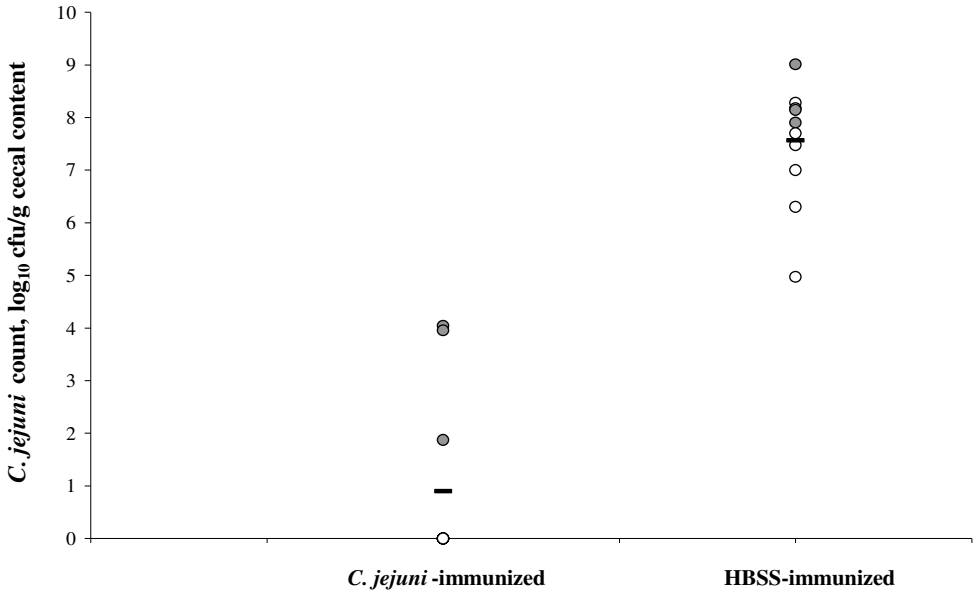

Figure 3.2 Individual cecal *C. jejuni* counts of two-week-old co-housed broiler seeders (●) and sentinels (○) receiving standard feed supplemented with 5% (wt/wt) egg yolk from either *C. jejuni*- (left) or HBSS-immunized (right) layers, from day six until the end of the experiment. At 10 days of age, seeder animals were inoculated with approximately 10^4 cfu of *C. jejuni* KC 40. Values are represented as $\log_{10}$ cfu/g cecal content.

In *in vivo* trial 2, which was performed in triplicate (data not shown), cecal *C. jejuni* numbers of inoculated broilers receiving hyper-immune egg yolks from layers immunized with the hydrophobic protein extract of *C. jejuni* were reduced by 2 logs (p = 0.002), compared to control animals (6.0 ± 1.8 vs. 8.0 ± 0.4 ($\log_{10}$ cfu/g cecal contents)). Moreover, transmission to non-seeder chicks was dramatically ($p < 0.001$) reduced (1.5 ± 2.5 vs. 7.0 ± 1.1 ($\log_{10}$ cfu/g cecal counts, averaged over all contact birds)). Together, this lead to a dramatic reduction ($p <$ 0.001) in the overall cecal *C. jejuni* count by $\log_{10}$ 4.4 for broilers receiving hyperimmune yolk (3.0 ± 3.2 $\log_{10}$ cfu/g cecal contents) compared to birds receiving control yolks (7.4 ± 1.0 $\log_{10}$ cfu/g cecal contents).

Identification of immunodominant *C. jejuni* KC 40 antigens

Comparing Western Blots from 2-dimensional separated *C. jejuni* proteins immuno-stained with IgY from egg yolk of *C. jejuni* whole cell lysate-immunized laying hens (A) with those stained with IgY from HBSS-immunized hens (B) revealed several immunodominant antigens, specifically recognized by anti-*C. jejuni* KC 40 egg yolk IgY (**figure 3.3**).

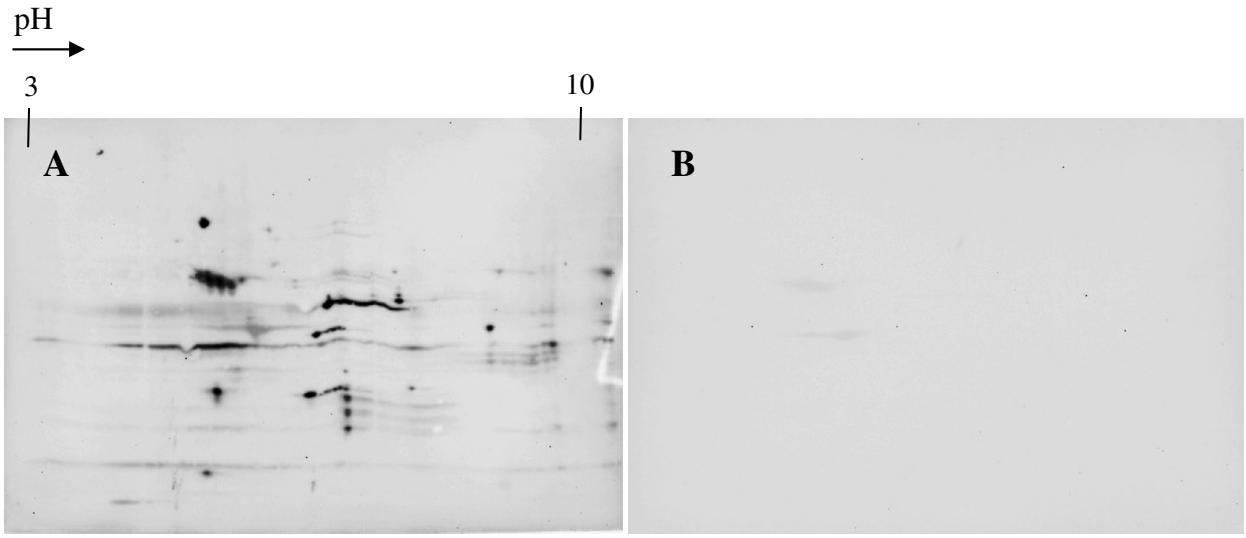

Figure 3.3 Western Blot of two-dimensionally separated *C. jejuni* KC 40 proteins, immunostained with purified immunoglobulin Y from egg yolks of laying hens immunized with *C. jejuni* KC 40 (A) or HBSS (B).

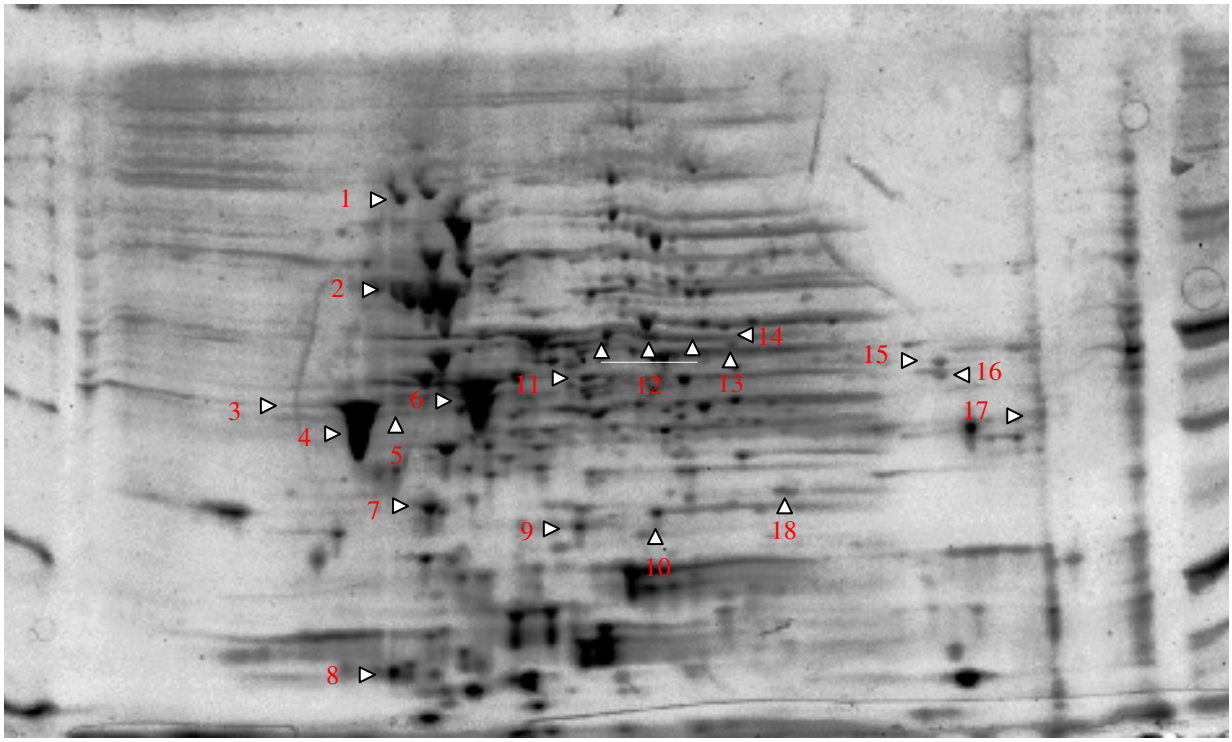

Figure 3.4 Two-dimensionally separated *C. jejuni* KC 40 proteins. Proteins indicated with a number were sequenced by mass spectrometry.

Spots of interest on the Western Blot, immunostained with purified IgY from yolks of laying hens immunized with *C. jejuni*, were linked to the 2-dimensionally separated *C. jejuni* proteins on the gel (**figure 3.4**), digested in peptides and used for mass spectrometric (MS) analysis. For each spot, multiple proteins were identified. Proteins with a Mascot score of < 110 were excluded from the study. MS analysis identified several of the immunodominant antigens of *C. jejuni* KC 40. Results of duplicate experiments are represented in **table 3.1** and **table 3.2**. Although in the second experiment not all protein spots could be identified, some additional differences were observed between the experiments: DnaK, glutamyl-tRNA synthetase, the hypothetical protein C8J_1250, oxidoreductase, 2-nitropropane dioxygenase, outer-membrane fibronectin-binding protein and a protein of the glutathionylspermidine synthase family protein were only found in the first experiment, while glycyl-tRNA synthetase alpha subunit, a probable thiol peroxidise, ribosomal protein L25, thioredoxin-disulfide reductase, the small unit of quinine-reactive Ni/Fe-hydrogenase, heat shock protein GrpE, hydrogenase expression/formation protein Hyp, dihydrodipicolinate synthase and cysteine synthase were only detected in the second experiment. The reason for this discrepancy is not clear but could at least in part be explained by the rather low resolution obtained during isoelectric focusing using a pH gradient of pH 3-10. A gradient of pH 4-7 may lead to a better resolution, resulting in less contamination after in-gel digestion but possibly protein loss.

Table 3.1 Predicted immunodominant *Campylobacter jejuni* antigens identified with HPLC-MS/MS (experiment 1)

Arrow no.	Identified *C. jejuni* proteins	Number of peptides	Protein score	Molecular weight (kDa)
1	Flagellar hook protein FlgE	45	1,389	-
2	Co-chaperonin GroEL	106	2,984	58
	Flagellin subunit FlaA	68	1,634	59
	Heat shock protein DnaK	12	564	12
3	Co-chaperonin GroEL	31	1,529	58
	Major outer-membrane protein MOMP	14	736	43
	Flagellin subunit FlaA	8	508	59
4	Major outer-membrane protein MOMP	113	3,001	43
	Translation elongation factor thermo unstable (Tu)	4	187	44
	Glutathionylspermidine synthase family protein	10	179	45
	Glutamate-1-semialdehyde 2,1-aminomutase	3	155	45
5	Major outer-membrane protein MOMP	22	903	43
	Translation elongation factor Tu	16	517	45
	Glutamate-1-semialdehyde 2,1-aminomutase	12	249	47
	Succinyl-CoA synthetase beta chain	9	246	42
	Translation elongation factor thermo stable (Ts)	9	235	40
	Cell division protein FtsZ	3	130	40
6	Translation elongation factor Tu	89	1,748	45

	Translation elongation factor Ts	63	688	40
	ATP-dependent Clp protease	12	351	46
	Major outer-membrane protein MOMP	4	205	43
	S-adenosylmethionine synthetase	7	187	44
7	Chemotaxis protein cheV	38	1,099	36
	Translation elongation factor Tu	11	477	45
8	FKBP-type peptidyl-prolyl cis-trans isomerase	13	745	21
	Inorganic pyrophosphatase	9	303	19
	Translation elongation factor Tu	2	186	43
9	Outer membrane fibronectin-binding protein CadF	2	152	36
10	Branched-chain amino acid aminotransferase	12	554	33
	Enoyl-(acyl-carrier-protein) reductase	6	344	30
	Putative methyltransferase	6	216	30
11	Methyl-accepting chemotaxis protein	26	552	41
	Succinyl-CoA synthetase beta chain	22	366	42
	Putative cysteine desulfurase	15	309	43
	Phosphoglycerate kinase	11	225	43
12	Conserved hypothetical protein	24	437	53
	Pyruvate kinase	11	365	54
	Glutamyl-tRNA synthetase	13	350	53
	ATP synthase F1, alpha subunit atpA	9	289	55
	Campylobacter trigger factor	11	260	51
	Aspartate ammonia-lyase	7	162	52
13	ATP synthase F1, alpha subunit atpA	19	1,007	55
	Glutamine-hydrolyzing GMP synthase	9	627	57
14	ATP synthase F1, alpha subunit atpA	25	1,013	55
	Peptide transport system substrate-binding protein	9	532	59
	Acetyl-CoA carboxylase	4	230	55
15	Putative secreted protease	10	801	49
16	Carboxyl-terminal protease ctpA	11	812	48
	Hypothetical protein C8J_1250 (flagellin modification protein pseA)	4	296	46
17	High affinity branched-chain amino acid ABC transport system, periplasmic binding protein livJ	2	219	40
	Oxidoreductase, 2-nitropropane dioxygenase	2	143	40
18	Putative UDP-glucose 4-epimerase	2	219	36

Table 3.2 List of immunodominant *Campylobacter jejuni* antigens (experiment 2)

Arrow no.	Identified *C. jejuni* proteins	Number of peptides	Protein score	Molecular weight (kDa)
1	Flagellar hook protein FlgE	25	1,035	-
2	Co-chaperonin GroEL	73	805	-
	Flagellin subunit FlaA	68	773	-
3	Flagellin subunit FlaA	9	312	-
4	Major outer-membrane protein MOMP	105	1,217	3.6
	Glutamate-1-semialdehyde 2,1-aminomutase	7	231	-
5	Major outer-membrane protein MOMP	14	359	46
	Cell division protein FtsZ	19	296	39
6	Translation elongation factor Tu	105	1,760	-
	Translation elongation factor Ts	30	656	40
	ATP-dependent Clp protease	2	155	46
7	Chemotaxis protein cheV	67	1,131	36
	Translation elongation factor Tu	13	468	45
	Glycyl-tRNA synthetase alpha subunit	5	157	24
8	Chemotaxis protein cheV	6	330	36
	Probable thiol peroxidase	5	233	19
	Translation elongation factor Tu	3	230	44
	Ribosomal protein L25	5	188	20
	Inorganic pyrophosphatase	7	172	19
	FKBP-type peptidyl-prolyl cis-trans isomerase	2	155	21
	Heat shock protein GrpE	4	141	21
9	Thioredoxin-disulfide reductase	4	341	34
	Quinine-reactive Ni/Fe-hydrogenase, small subunit	5	277	40
10	Enoyl-(acyl-carrier-protein) reductase	5	170	30
11	Translation elongation factor Tu	3	146	-
12	Aspartate ammonia-lyase	5	120	-
16	Carboxyl-terminal protease ctpA	19	915	48
17	High affintiy branched-chain amino acid ABC transporter	8	419	40
	Hydrogenase expression/formation protein HypE	3	153	41
18	Dihydrodipicolinate synthase	6	194	33
	Cysteine synthase	6	140	33
	Putative UDP-glucose 4-epimerase	7	110	36

DISCUSSION

Immunizing laying hens and subsequently collecting their eggs is a cheap, straightforward and non-invasive method to obtain high amounts of specific antibodies (Mine & Kovacs-Nolan, 2002; Dias da Silva & Tambourgi, 2010). In our study, *C. jejuni*-specific IgY was dramatically induced in egg yolk of hens immunized with a *C. jejuni* lysate, resulting in titers of up to 1:16,000 as determined by ELISA (results not shown). In contrast, specific IgY in egg white and specific IgA in egg white and yolk were not significantly induced after

immunization and were present at only marginal concentrations in both groups. These observations are in line with those of Dohms *et al*. (1978), where transfer of radioactive labled immunoglobulins (IgA, IgM and IgY) from the turkey hen into the egg was examined, indicating that only transfer of IgY to egg yolk is biologically relevant in the overall transfer of immunoglobulins into eggs.

In the first *in vivo* trial, using the whole cell lysate of *C. jejuni*, broiler chicks were provided with feed supplemented with (hyperimmune) egg yolks from day seven onward. At 10 days of age three out of 11 birds were orally inoculated with approximately 8×10^3 cfu *C. jejuni*. Colonization of these seeder animals is followed by transmission of *C. jejuni* to the remainder of the birds. Three days after inoculation, cecal *C. jejuni* numbers of seeder birds receiving hyperimmune egg yolk were significantly reduced compared to control seeders without IgY (by over 5 logs). Moreover, none of the non-seeder birds were colonized with *C. jejuni*, while chicks receiving control eggs carried high (ca. $\log_{10}$ 7 cfu/g) bacterial numbers in their ceca. It is not clear at what site (the cecum or more proximal in the GI tract) the IgY fraction was active, but clearly it captured invading *C. jejuni* bacteria, disabling them to replicate and transmit to other birds. In addition, specific IgY increased bacterial binding of *C. jejuni* to chicken intestinal mucus, suggesting that it promotes bacterial uptake in the mucus layer for enhanced mucosal clearance. Because several of the immunodominant antigens were identified to be associated with the bacterial outer-membrane, a second (analogous) *in vivo* trial was performed using only the hydrophobic protein fraction of *C. jejuni*. Although the effects observed in this study were less pronounced compared to the results obtained with the whole cell lysate (probably due to the higher inoculation dose of the seeders (3×10^4 cfu vs. 8×10^3 cfu)), hyperimmune yolks reduced cecal bacterial counts of seeders by 2 log and dramatically reduced *C. jejuni* transmission to contact birds. Previous passive immunization studies with *Salmonella* by other researchers indicated no effect at all on cecal colonization of broilers receiving feed supplemented with 5% (wt/wt) freeze-dried egg yolk powder containing anti-*Salmonella* spp. IgY (Chalghoumi *et al*., 2009b). These authors suggested that the antibodies were denatured and degraded along the GI tract, thereby reaching the ceca at insufficient concentrations. In our study, egg yolks were administered as such, because they contain antibacterial substances other than IgY and to exclude degradation during freeze-drying. Several reasons could explain the discrepancy between the two studies and include (1) bacterium-specific differences between *Salmonella* spp. and *C. jejuni* or differences in (2) IgY-formulations. Indeed, the reduction in cecal *C. jejuni* counts we obtained in our study could indicate that egg yolks form a protective matrix, allowing IgY to survive the digestive

enzymes and the low pH along the GI tract (Mine & Kovacs-Nolan, 2002); (3) experimental set-up, since in the study by Chalghoumi *et al*. (2009b) all birds were challenged with *Salmonella*, while we used a seeder experiment and age of the animals at challenge; and (4) immunization protocol, resulting in higher IgY titers in the hen egg yolks in our study.

Western Blot analysis followed by HPLC-MS/MS and BLAST analysis identified the most likely *C. jejuni* proteins responsible for the induction of specific IgY. Because a *C. jejuni* whole cell lysate was used, specific IgY could be induced against both membrane-bound as well as cytoplasmic proteins. Although our obtained results are fairly consistent with those reported in other studies examining antigenic proteins of *C. jejuni*, there was a striking absence in our study of Omp18, Cme, Cja and PEB proteins (Shoaf-Sweeney *et al*., 2008). These differences might be in part explained by *C. jejuni* strain differences. In addition, it is not clear to what extent repeated immunization of chickens in their breast muscle could lead to a differential immune respons compared to that observed during (natural) commensal colonization with *C. jejuni*.

A first group of proteins that reacted with the purified egg yolk IgY are flagellar proteins: the flagellar hook protein FlgE and the major flagellin FlaA. Both proteins are known to be immunogenic and are needed for full motility of *C. jejuni* (Shoaf-Sweeney *et al*., 2008, Hermans *et al*., 2011b).). FlgE mediates flagellar assembly and protein secretion in *C. jejuni* (Shoaf-Sweeney *et al*., 2008). In addition, mutation of the highly conserved FlaA results in a colonization defect (Wassenaar *et al*., 1993). These results are consistent with Shoaf-Sweeney *et al*. (2008) who reported the presence of maternal antibodies reacting with these flagellar proteins in the serum of laying hens. Despite these flagellar proteins were shown to be highly immunogenic in this study, specific IgY did not affect *C. jejuni* motility.

The major outer-membrane protein (MOMP) of *C. jejuni*, encoded by the *porA* gene, was identified in several gel pieces, pointing to its abundance in the *C. jejuni* proteome (Islam *et al*., 2010). In addition to being immunogenic (Huang *et al*., 2007), MOMP is involved in adhesion and transmembrane ion transport in *C. jejuni* (Islam *et al*., 2010). However, MOMP is extremely genetically diverse and several conformational epitopes have been implicated in the induction of protective immunity (Huang *et al*., 2007), thereby possibly hindering its use in vaccine applications.

An outer-membrane fibronectin-binding protein with an apparent mass of 36 kDa was presumed to be CadF (*Campylobacter* adhesion factor). CadF is a highly prevalent protein in *C. jejuni* where it is important for adhesion to chicken epithelial cells and cecal colonization

of this host (Ashgar *et al*., 2007). This protein was, however, identified in only one of duplicate experiments with only few peptides matching, pointing to a contamination product.

Methyl-accepting chemotaxis (MCP) proteins are transmembrane receptors for chemotactic stimuli (Hermans *et al*., 2011b). The *C. jejuni* genome encodes several MCP proteins and it is not clear which MCP protein, with an apparent mass of 41 kDa was identified in this study. Attached to these transmembrane receptors is the *C. jejuni* chemotaxis protein V (CheV), a coupling protein involved in transducing chemotactic signals in *C. jejuni* (Lertsethtakarn *et al*., 2011), which was also identified in this study.

Another immunodominant protein was the branched-chain amino acid ATP-binding cassette transport protein livJ, a periplasmic binding protein probably involved in transporting amino acids into the bacterial cell (Ribardo & Hendrixson, 2011) and crucial for chick colonization (Hermans *et al*., 2011b). An additional role for LivJ in the interaction of *C. jejuni* with the chick cecum was suggested by Ribardo & Hendrixson (2011).

ATP synthase is abundantly present and highly conserved among bacteria (Kovach *et al*., 2011). The protein is associated with the membrane where it performs its role in energy metabolism. In this study, the alpha subunit of ATP synthase F1 (AtpA) was found to be immunogenic in *C. jejuni*, as already reported for *C. concisus* (Kovach *et al*., 2011).

Most of the identified antigens in this study are proteins, mainly located in the bacterial cytoplasm where they perform their respective functions. Some of these antigens may nevertheless be interesting candidates for vaccine development.

GroEL and GrpE are heat shock proteins that play a crucial role in the *C. jejuni* stress response and the former has also previously been shown to be a immunodominant antigen of *C. jejuni* (Zhang *et al*., 2012). GroEL, belonging to the 60-kDa family, is an extremely conserved protein (Thies *et al*., 1999), and although mainly located in the bacterial cytoplasm, in *Salmonella* (and other bacteria) it is suggested to be expressed on the cell surface as well since GroEL has been shown to mediate *Salmonella* adhesion (Tsugawa *et al*., 2007).

The highly conserved translation elongation factor thermo unstable (EF-Tu) was found to be abundantly present in the *C. jejuni* lysate, which is in line with observations in other bacteria (Nieves *et al*., 2010). Despite its cytoplasmic role during protein synthesis, EF-Tu was shown to be translocated to the surface in several bacteria, where it mediates adhesion and invasion of host cells (Nieves *et al*., 2010). Immunization of mice with *Burkholderia pseudomallei* EF-Tu resulted in a potent immune response that was partially protective against melioidosis.

Finally, a putative secreted carboxyl-terminal protease of *C. jejuni*, CtpA, was identified. This protein has been shown to be highly prevalent and conserved in *Burkholderia mallei*, another Gram-negative bacterium (Bandara *et al*., 2008).

Taken together, this study reports some very promising observations, but much more research is needed before a commercial product can be promoted. First of all, the observations from this study need to be validated using more animals. Second, it needs to be established whether this protective effect is maintained over periods exceeding three days post-inoculation. Also, the immunization protocol and the yolk dose in the feed need to be optimized. Further research will also have to elucidate whether this strategy is capable of providing cross-protection against heterologous *C. jejuni* strains. Eventually, this may lead to the development of a passive immunization strategy that can succesfully control *C. jejuni* in chickens. If the relevant protective antigens of *C. jejuni* could be identified and studies regarding conservation and prevalence among *C. jejuni* strains are conducted, subunit vaccines able to target a wide range of *C. jejuni* strains could be developed for passive immunization applications.

To conclude, we here demonstrate for the first time that feeding broilers IgY-rich yolks from hens repeatedly immunized with *C. jejuni* (using either a whole cell lysate or with the hydrophobic protein fraction) through their feed dramatically reduces both *C. jejuni* numbers in the ceca after challenge with the homologous strain and transmission to non-inoculated contact birds, providing a solid base for further research regarding passive immunization to control *C. jejuni* colonization in broiler flocks.

Acknowledgements

This work was financed by a grant of the Federal Public Service for Health, Food Chain Safety and Environment (FOD, Brussels, Belgium): project RT08/8-CAMPOUL. We would like to thank Hanne Vereecke and Nathalie Van Rysselberghe for their excellent technical assistance during the *in vivo* trial.

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CHAPTER 4: GENERAL DISCUSSION

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Gastro-intestinal illness caused by food-borne bacteria puts a serious public health burden worldwide, with *Campylobacter* spp. and *Salmonella enterica* as the zoonotic pathogens with the largest impact (EFSA, 2012). Recent research in this area resulted in the development of several intervention measures that can reduce the prevalence of these bacteria in food animals. Poultry very often is colonized with *Campylobacter* spp., but in contrast to some other bacteria (e.g. vaccination against *Salmonella*), no effective strategy exists to reduce the prevalence of this zoonotic pathogen in poultry production. As a consequence, *Campylobacter* is today the number one cause of bacterial-mediated gastro-intestinal disease in humans, with *C. jejuni* as the most important causative agent (Hermans *et al*., 2011a).

C. jejuni is highly adapted to the chicken gut and colonized birds may carry a high bacterial load, especially in their ceca, without developing clinical signs. Broiler flocks are typically colonized from two weeks of age onward up to slaughter, resulting in high numbers of contaminated carcasses after processing (Hermans *et al*., 2011b). Since the cecum is the only amplification point of *C. jejuni* throughout the entire food chain, on-farm intervention measures that reduce colonization of the ceca may have a significant impact on the number of campylobacteriosis cases in humans.
The general scientific aim of this PhD thesis was to develop an intervention measure that consistently reduces *C. jejuni* numbers in the ceca of broiler chickens by a thousand-fold, which would lead to a reduction in the number of human campylobacteriosis cases by 90%.

Contribution of fatty acids to control *C. jejuni* in broiler chickens

Concerns regarding antimicrobial resistance resulted in the ban of the use of antimicrobial agents in poultry as growth promoters by the European Union (Dibner & Richards, 2005). Therefore, other strategies will have to be developed for controlling *C. jejuni* in poultry.

For this purpose, the use of short-chain fatty acids (SCFA) and medium-chain fatty acids (MCFA) (caproic, caprylic, capric and lauric acid) has been extensively studied. The anti-bacterial mode of action of these acids is not precisely defined. It is believed that acids diffuse into bacterial cells in the un-dissociated form and dissociate in the more alkaline bacterial cytoplasm. The excess in ions formed would eventually kill the bacterium by depleting ATP (Russel, 1992; Sun *et al*., 1998). This might indeed explain the bactericidal, dose-dependent

activity of the tested MCFA observed at pH 6 in our study and the reduced sensitivity of acid-tolerant bacteria toward the antibacterial effects of organic acids (Van Immerseel *et al*., 2006), but does not explain why MCFA are more active compared to SCFA against several bacteria, including *C. jejuni* and *Salmonella* (Boyen *et al*., 2008). Probably, other factors such as composition of the backbone, hydrophobicity and the acid dissociation constant pKa of the acids will also determine the antimicrobial activity toward a given bacterium (Van Immerseel *et al*., 2006). Our observed MIC values for MCFA toward *C. jejuni* were markedly lower than those obtained for *Salmonella* (Boyen *et al*., 2008). However, while for *Salmonella enterica* serovar Enteritidis some reduction has been observed in cecal colonization of chicks fed caproic acid (Van Immerseel *et al*., 2004a), based on the results of this thesis it seems that MCFA administration to drinking water or feed is not capable to target *C. jejuni* in the chick cecum but can at most exert a bactericidal effect more proximal in the gastro-intestinal (GI) tract. For *Salmonella* Enteritidis, it was shown that MCFA reduce the expression of *hilA*, a key regulator of epithelial cell invasion, thereby possibly explaining the observed reduction in cecal *Salmonella* counts in chicks fed MCFA (Van Immerseel *et al*., 2004a). For *C. jejuni*, such events have not been identified.

Medium-chain fatty acids are not effective in the chick cecum

MCFA can be administered preventively to broiler flocks, from day-of-hatch, but more cost-efficient would be to incorporate them into the feed and/or drinking water prior to slaughter. Coating MCFA on micro-beads to prevent premature absorption and/or degradation of the acids in the upper GI tract is crucial in such applications, allowing them to reach the ceca (Van Immerseel *et al*., 2004b), which is the predominant site for *C. jejuni* colonization (Beery *et al*., 1988). However, coated and free MCFA-supplemented feed at 1% (wt/wt), as well as drinking water treated with a MCFA-in-water emulsion at a 0.4% (vol/vol) dose did not reduce cecal *C. jejuni* counts in experimentally inoculated chicks when administered three days before euthanization. Although it was not validated in these studies whether the active fatty acid concentration that reached the ceca was high enough for bactericidal activity, *in vitro* bactericidal concentrations did not prevent growth and survival when injected directly into the ceca. Therefore, developing proper coating materials to deliver larger acid amounts in the ceca seems useless. Alternatively, *C. jejuni* colonization in broilers could be prevented by administering MCFA from day-of-hatch up to slaughter. However, in seeder experiments, preventive administration of MCFA-treated drinking water did not reduce cecal *C. jejuni* counts of inoculated chicks. In addition, the spread of *C. jejuni* from experimentally

inoculated to non-inoculated birds was not prevented, in line with findings of Hilmarsson *et al*. (2006) and van Bunnik *et al*. (2012). Drinking water of broilers is believed to play a key role in *C. jejuni* transmission (Messens *et al*., 2009; Hermans *et al*., 2011b). Apparently, the reduced pH of the treated drinking water, which increased the bactericidal potency of the acids (Hermans *et al*., 2010) and even prevented the drinking water to become contaminated with *C. jejuni*, was not sufficient to prevent bird-to-bird transmission, which most likely happened by the fecal-oral route.

Conflicting results

Several contradictory observations to ours have been reported by others, suggesting that MCFA applications might be more effective in controlling *C. jejuni* in poultry then our results suggest. For instance, it was found that the ability of the bacterium to colonize the broiler cecum can be reduced by supplementing medium-chain fatty acids to the broiler feed from day-of-hatch (Solis de los Santos *et al*., 2008a) or even completely prevented with a combination of formic acid and sorbate (Skånseng *et al*., 2010). Additionally, therapeutic administration of caprylic acid to the feed decreased cecal *Campylobacter* numbers by multiple logs both in 15-day-old (Solis de los Santos *et al*., 2008b) and market-aged (Solis de los Santos *et al*., 2009) broilers. The reason for these contradictory observations is not clear, but may be explained by 1) differences in formulation of the acid in the feed and/or drinking water, allowing it to reach the ceca at higher concentrations in some studies, although our results with the cecal loop model suggest that MCFA are not effective in targeting *C. jejuni* in the cecum even at elevated concentrations; 2) *C. jejuni* strain-specific properties, as we inoculated the chicks with a highly invasive strain that might be less susceptible toward the effects of MCFA in the broiler cecum compared to less invasive strains; or 3) other factors, such as differences in genetic background of the birds used or differing dietary influences on cecal biochemistry.

Protective niche theory

Our findings made us conclude that MCFA can neither target *C. jejuni* in the chick cecum, nor prevent bird-to-bird transmission with *C. jejuni* after the first bird is colonized. Thus, the use of fatty acids will most likely not result in either a substantial decrease in the number of *C. jejuni*-colonized animals or the number of viable bacteria in the ceca of colonized animals. Apparently, *C. jejuni* harbours a “protective niche”, rendering the bacteria unresponsive to the bactericidal effect of organic acids observed *in vitro*. One of the major questions remaining to

be answered is why there is such a big discrepancy between *in vitro* and *in vivo* observations for the tested compounds. After all, the acid concentrations needed to inhibit growth of or to kill *C. jejuni* are approximately 10-fold lower compared to other bacterial pathogens, such as *Salmonella enterica*. In contrast, *in vivo* concentrations that are higher compared to those successfully applied to moderately reduce *Salmonella enterica* serovar Enteritidis numbers in chicks, failed to induce any effect at all against *C. jejuni*. Intestinal mucus was shown to reduce the anti-*C. jejuni* activity of both SCFA (Van Deun *et al*., 2008) and MCFA, indicating that the chicken intestinal mucus layer, which covers the entire surface of the chicken intestinal tract (Forstner *et al*., 1995), clearly plays a protective role. Possibly, it forms a physical barrier between the bacterium and the acid, because *C. jejuni* is found deep in the cecal crypts within the mucus layer (Beery *et al*., 1988). Thus, whereas the chicken intestinal mucus layer usually is regarded as part of the innate host defense system and thus an anti-bacterial barrier (Forstner *et al*., 1995), it seems that in the case of *C. jejuni* it forms a protective and supportive environment making it hard to eradicate this bacterium from the chicken intestinal tract, once colonized. Although some chicken intestinal mucus factors promoting *C. jejuni* colonization have been identified (such as providing all necessary nutrients for optimal growth, promoting chemotaxis and enhanced secretion of the *Campylobacter* invasion antigens), it is not clear how the intestinal mucus layer contributes to increased resistance of *C. jejuni* against fatty acids.

Reducing the colonization threshold

This raises the question whether MCFA are capable of exerting a bactericidal effect in more proximal sites of the GI tract, thereby possibly preventing cecal *C. jejuni* colonization in chicks. Indeed, we demonstrated that MCFA-treated drinking water at a 0.4% (vol/vol) dose from day-of-hatch increased the colonization threshold of individually housed broilers when inoculated with low (2.8×10^2 cfu) to moderate (2.2×10^3 cfu) *C. jejuni* numbers, which is in line with Chaveerach *et al*. (2004) and with observations made using an in-feed approach (van Gerwe *et al*., 2010). MCFA-treatment reduced the pH of the drinking water from neutral to 5.4, thereby increasing the bactericidal potency of the acids. This reduced pH might aid in killing *C. jejuni* in the crop and stomach, thereby preventing a bacterial population to reach and colonize the cecum. Indeed, Byrd *et al*. (2001) observed reduced *C. jejuni* counts in crops of broilers receiving drinking water supplemented with 0.44% lactic acid. Again, however, in our study, MCFA-treated drinking water did not result in an overall reduced cecal *C. jejuni* load of co-housed chicks, five days after inoculation of seeders with this moderate (3×10^3

cfu) *C. jejuni* dose. Possibly, *C. jejuni* transmission between birds was responsible for a reduction in the apparent minimal infective dose (Conlan *et al*., 2011), because fecal *C. jejuni* shedding by colonized birds increases the contamination pressure for the remainder of the animals dramatically (Hermans *et al*., 2011b). Under these conditions, MCFA are not capable anymore to prevent *C. jejuni* colonization of other animals. It can, however, not be ruled out that MCFA are capable of preventing cecal colonization of co-housed birds upon inoculating seeders with lower *C. jejuni* numbers.

Conclusion

Although not effective in the ceca, MCFA prevent *C. jejuni* survival in treated drinking water and may kill *C. jejuni* to some degree in more proximal sites of the GI tract. On-farm, however, birds are under a constant contamination pressure from the outside environment and each other. Therefore, bacteria that survive and reach the ceca may eventually result in a colonized broiler, acting as a transmission source after a stable colonizing population has been reached in the ceca. The rapid fecal-oral spread after the first bird is colonized will then eventually result in the whole flock being colonized at slaughter age. At this point, it seems that the use of MCFA is useless.

Nevertheless, supplementing MCFA to the drinking water of broiler chicken flocks throughout the whole rearing period, together with proper implementation of biosecurity measures (to limit *C. jejuni* exposure) could be helpful in preventing these flocks to become colonized. Moreover, because drinking water and drinking water equipment contaminated with *C. jejuni* contribute to the rapid transmission of *C. jejuni* in a broiler flock (Messens *et al*., 2009; Hermans *et al*., 2011b), the rapid killing of *C. jejuni* in MCFA-treated water at the applied dose inhibits *C. jejuni* transmission via the drinking water, and excludes drinking water as a possible way for *C. jejuni* to enter and colonize a flock, which are additional advantages over in-feed addition of MCFA. In addition, in a very recent study, it was demonstrated that MCFA administration significantly reduced indirect transmission between spatially separated chicks (van Bunnik *et al*., 2012), suggesting that drinking water acidification may be capable in reducing between-flock transmission. In any case, it seems that the most effective way to benefit from the antibacterial effects of MCFA is their preventive administration to increase the number of viable *C. jejuni* bacteria needed for successful colonization of broilers and therefore the probability of *C. jejuni* to enter a flock. Large-scale field trials are needed to examine whether such a reduced colonization threshold,

imparted by treating the drinking water with MCFA, is capable to prevent cecal *C. jejuni* colonization in chicks under farming conditions.

Contribution of plant-derived antimicrobial compounds to control *C. jejuni* in broiler chickens

Screening experiments of several plant-derived compounds were performed at pH 6, approaching the mean cecal pH of broilers during their life-cycle (van der Wielen *et al*., 2000), which compensates for any pH-dependent effect. Among the plant-derived antimicrobial compounds (PDAC) tested, the cinnamon-oil ingredient *trans*-cinnamaldehyde (CIN) and the green tea polyphenol epigallocatechin gallate (EGCG) were the most effective (MIC of 125 μ*M*) at preventing *C. jejuni* growth. In addition, for CIN a lower minimal bactericidal concentration was observed compared to EGCG. Also Friedman *et al*. (2002) found *trans*-cinnamaldehyde to be one of the most active substances toward *C. jejuni in vitro* out of a more extensive selection of compounds. Hence, CIN seemed a very promising candidate as a feed additive to reduce *C. jejuni* colonization in poultry and was therefore tested *in vivo*. Using a seeder model, we showed that CIN is not able to prevent transmission and colonization and does not reduce cecal *C. jejuni* counts at the concentrations tested, even when the compound was micro-encapsulated. *In vitro* incubation of *C. jejuni* with CIN at doses of 250 μ*M* or higher reduced bacterial counts below the detection limit within eight hours post-inoculation, but viable bacteria were detected 24 hours pi. The reason for this re-emergence is not clear, but might possibly be explained by the development of a resistant subpopulation of *C. jejuni* (although unlikely after such short incubation times), or the ability of *C. jejuni* to metabolize or degrade *trans*-cinnamaldehyde. In addition, in pigs, CIN is degraded very quickly along the GI tract (Michiels *et al*., 2008). Whether this is also the case in chicks is not clear, but at least some of these events may possibly explain the ineffectivity of CIN *in vivo*. However, direct injection in chicken ceca of CIN concentration of multiple times the MIC did not reduce *C. jejuni* counts two or 24 hours after injection, thereby excluding all these possibilities and suggesting a rather similar reason for not being effective as observed for fatty acids.

Influence of the model and *C. jejuni* strain used on experimental outcome

For all *in vivo* trials, a highly invasive and efficiently colonizing *C. jejuni* strain (*C. jejuni* KC 40, isolated from chicken feces and passaged twice in broilers) was used, resulting in a highly reproducible model in which all animals were colonized, with very high cecal bacterial counts, 24 hours after inoculation. Given these characteristics of *C. jejuni* KC 40, it could be possible that inoculation with this strain is too overwhelming for allowing any potentially beneficial effect to be noticed. However, preliminary experiments using a low invasive *C. jejuni* strain (KC 59.1) revealed similar (even slightly higher) colonization levels with the absence of a positive effect when using a mixture of MCFA (results not shown). Moreover, because control measures should be able to target most, if not all, *C. jejuni* strains, the choice for working with *C. jejuni* KC 40 is definitely warranted.

Second, on-farm birds are under a constant contamination pressure from the surrounding environment, while in our models birds were challenged with *C. jejuni* only once. On the other hand, in some models, (all) animals were inoculated with high doses of the *C. jejuni* strain. It is doubtful that such events will take place on-farm. However, based on the literature and experiments conducted at this laboratory it seems that *C. jejuni* will be transmitted to all animals shortly after the first animal is colonized, even if only a few birds are challenged with relatively low *C. jejuni* doses. Indeed, it was estimated that all (20,000) chicks in a flock will be colonized seven days after *C. jejuni* is detected in the first bird (Stern *et al*., 2001). Therefore, it is doubtful that less aggressive models could have led to more satisfactory results and the absence of effectiveness is probably not attributable to the models used. The marked reduction in cecal *C. jejuni* counts (over three logs) obtained in our therapeutic model using the macrolide tylosin definitely supports this hypothesis.

Urgent need for alternative strategies

Due to practical concerns, only a limited number of fatty acids and plant-derived antimicrobials were tested *in vivo*. An *in vitro* fermentation model mimicking the broiler's cecal environment could be helpful to examine whether our observations with the studied compounds can be extrapolated to all fatty acids and PDAC. Nevertheless, from our observations it is clear that caproic acid, caprylic acid, capric acid and *trans*-cinnamaldehyde at relevant doses are not capable to consistently reduce the cecal *C. jejuni* load of broiler

chickens by at least three logs. Therefore, it has to be assessed whether other on-farm intervention measures are capable of reaching this goal.

For this purpose, several strategies could be used, including the use of bacteriophages, competetive exclusion, bacteriocins, vaccination and passive immunization, as summarized by Hermans *et al*. (2011a).

The use of bacteriophages seems very promising but has the disadvantage of being strain-specific. Therefore, the reducing potential of a specific phage will differ from one region to another depending on the prevalent *C. jejuni* strains. Although the problem of this specificity can be reduced by using a bacteriophage cocktail, the development of a universal bacteriophage product seems not feasible. In addition, a relatively high degree of bacteriophage resistance has been reported with this strategy. However, bacteriophage-resistant phenotypes are poor colonizers and in absence of selective (bacteriophage) pressure, they regain their efficient colonization and phage-sensitive status (Scott *et al*., 2007).

Competitive exclusion (CE) cultures could provide the ideal intervention measure if they share the same colonization site as *C. jejuni*. By blocking interaction/colonization sites and competing for essential nutrients they could prevent *C. jejuni* from colonizing their protective cecal niche. However, the reported observations using CE are not very promising (Hermans *et al*., 2011a) and more research is needed regarding both the chicken and *C. jejuni* factors involved in this interaction. This may substantially reduce the number of relevant candidates, eventually leading to the identification of CE cultures effective in preventing *C. jejuni* from colonizing its cecal niche. Bearing in mind our "cecal niche theory", such an approach could render *C. jejuni* more susceptible to the antibacterial properties of organic acids and PDAC, making their potential contribution to *C. jejuni* control in chicks not so limited after all. To test this hypothesis, studies regarding the simultaneous use of CE cultures (to prevent *C. jejuni* colonization) and organic acids or PDAC (to enhance mucosal killing of *C. jejuni*), are needed.

The use of purified bacteriocin from bacteriocin-producing *Enterococcus* isolates seems very promising. Svetoch & Stern (2010) reported dramatic reductions by over 6 logs in cecal *C. jejuni* counts. However, there is a lack of knowledge concerning potential development of resistance to bacteriocins in *C. jejuni*. Although *in vitro* emergence of bacteriocin-resistance in *C. jejuni* was recently shown to be rather limited, it is currently not known whether high selection pressure could promote bacteriocin emergence *in vivo* (Hoang *et al*., 2011).

Finally, also immunization-based approaches should be considered for *C. jejuni* control. Although in chickens *C. jejuni* is able to invade epithelial cells and colonize the spleen (Van Deun *et al*., 2008), its interaction with this host is believed to be of commensal nature, devoid of any inflammatory response of the chicken intestinal wall and clinical signs during colonization (Hermans *et al*., 2011c). Therefore, direct vaccination of young broiler chickens is not expected to induce a potent immune response, although some reduction (log_{10} 1.4 cfu/g) has been observed in cecal *C. jejuni* numbers of birds vaccinated with a *Salmonella* Typhimurium mutant expressing CjaA (Buckley *et al*., 2010). In addition, manual vaccination of all broiler chicks in a flock, *per os* and definitely through injection, would be very time-consuming. Alternatively, as often performed for vaccinating breeder chickens against *Salmonella*, the vaccine could be administered via the drinking water. To our knowledge, however, no reports have been published where such an approach was successful in reducing cecal *C. jejuni* counts in broiler chicks.

Passive immunization: a promising alternative approach?

The negative features of direct vaccination (lack of an appropriate immune response and cost-effectiveness) might be circumvented by hyper-immunizing laying hens and subsequently collecting their eggs. The induced immunoglobulins (mainly IgY) are transported to and accumulated in the egg yolks of these animals. By simply collecting these IgY-rich eggs and administering the yolks to the feed of broiler chickens, *C. jejuni* colonization may be prevented or reduced. Passive immunization thus seems very promising and was therefore evaluated as a possible alternative to in-feed antibiotics to combat *C. jejuni* colonization in chickens.

Passive immunization has been shown to be effective against a wide variety of disease-causing bacteria, including *H. pylori* (Shimamoto *et al*., 2002). In a study from 1997 (Tsubokura *et al*., 1997), it was shown that passive immunization might also have a beneficial contribution to reduce *C. jejuni* shedding in poultry. However, this is the only published report concerning this matter and this technique needs optimization.

For this purpose, during this thesis, commercial laying hens were repeatedly immunized with, either a whole cell lysate or the hydrophobic protein fraction of, *C. jejuni* KC 40, using Freund's Complete (first immunization) and Incomplete (booster immunizations) Adjuvans for inducing a potent immune response. Five weeks after the first immunization, egg yolks started to accumulate high titers of specific IgY, as judged by enzyme-linked immunosorbent

assay, which were maintained for several weeks (monitored up to the end of the *in vivo* trial). Administering these egg yolks to the feed of chicks at 5% (wt/wt) tremendously lowered cecal *C. jejuni* colonization of seeder animals upon homologous inoculation and (almost) completely prevented transmission to contact birds. *In vitro* analyses showed that purified IgY promoted *C. jejuni* binding to intestinal mucus, which may be responsible for increased mucosal clearance of *C. jejuni* bacteria. In contrast, IgY did not alter motility of the homologous *C. jejuni* strain at these concentrations, which is rather unexpected given the high immunogenicity of *C. jejuni* flagella (Sahin *et al*., 2001; Shoaf-Sweeney *et al*., 2008). In addition, Shoaf-Sweeney *et al*. (2008) did show reduced motility of a homologous, but not that of a heterologous strain by maternal antibodies *in vitro*.

Two-dimensional gel electrophoresis followed by Western Blot analysis and electro-spray ionization-mass spectrometry identified several of the immunodominant antigens of *C. jejuni* KC 40, including the flagellar hook protein FlgE2. The immunodominance of this flagellin protein is in line with the reported immunogenicity of the *C. jejuni* flagellum (Sahin *et al*., 2001; Shoaf-Sweeney *et al*., 2008) but raises further questions why purified IgY did not affect *C. jejuni* KC 40 motility in our *in vitro* experiments.

Conclusions and what to do in the mean time?

To conclude, although some on-farm approaches seem promising and deserve further attention, there is still no effective and reliable commercial intervention measure available to date. As a consequence, the number of human campylobacteriosis cases remains strikingly high (EFSA, 2012).

The results of this thesis indicate that especially the use of passive immunization is very promising to reduce *C. jejuni* colonization in poultry and should deserve full further attention. We suggest that recombinant production of conserved immunodominant antigens, followed by studying them in passive immunization experiments could lead to the identification of *C. jejuni* antigens crucial for chick colonization. Promising candidates for this purpose may include GroEL, EF-Tu, AtpA, CtpA and MCPs. Increased knowledge on the interaction of *C. jejuni* with its chicken host could then be exploited for further fine-tuning of passive immunization as an effective intervention measure to control *C. jejuni* in poultry. Protective antigens could be used to hyperimmunize chicks, collecting their eggs and feeding them to broilers. Such an approach allows to pool eggs from different hens and/or from one hen at

different time intervals, thereby reducing batch-to-batch variation. However, more research is needed to validate whether such an approach would be feasible on a large scale. Alternatively, antibodies against these protective antigens, if any, could be identified and recombinantly and cost-efficiently produced in plants, which can be mixed through the chicken feed. However, much more research is needed to determine whether such an approach is realistic.
It is not clear whether the current approach of preventively feeding broilers with 5% (wt/wt) egg yolk would be cost-effective. And if so, to what extent would farmers be willing to pay the price to prevent their flocks from colonization with a commensal bacterium that does not lead to economical losses, and as such does not give them any advantage? This urges the need for a kind of "bonus system" where farmers are encouraged to consistently deliver *Campylobacter*-free batches.

In the meantime, it is of utmost importance to inform the customer about the risk posed by *Campylobacter*-contaminated chicken meat products and to enhance biosecurity measures on the farm and during slaughter. Proper implementation of on-farm measures, such as the use of a footbath before entering the broiler house, cleaning and disinfection of farm equipment and insect and rodent control programs should be able to reduce the prevalence of *C. jejuni* in broiler flocks, while proper cleaning and disinfecting of abattoir equipment should reduce the likelihood for cross-contamination. Such measures can be readily and rather cost-effectively be implemented and should be aimed to keep the number of *Campylobacter*-contaminated chicken meat products under control. Possibly, these measures could be combined with treating the drinking water of broiler flocks with medium-chain fatty acids to increase their *C. jejuni* colonization treshold, but large-scale field trial are needed to prove the effectivity of this approach. It is very doubtful, however, that these measures alone would be sufficient to dramatically reduce the *C. jejuni* prevalence in poultry.

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SUMMARY

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Campylobacter jejuni is the most common cause of bacterial-mediated diarrheal disease in humans worldwide and poses a serious health burden. Poultry is considered the most important source for campylobacteriosis in humans. *C. jejuni* is highly adapted to the chicken gut and colonized birds carry a very high bacterial load, especially in their ceca, without developing any clinical signs. Broiler flocks are typically colonized from two weeks onward up to slaughter, resulting in a high number of contaminated carcasses after processing which can transmit the pathogen to humans. Since the cecum is the only amplification point of *C. jejuni* throughout the entire food chain, on-farm intervention measures that reduce colonization of the ceca may have a significant impact on the number of campylobacteriosis cases in humans. However, at present, no effective strategy exists to clear *C. jejuni* from broiler flocks. As a consequence, the incidence of campylobacteriosis in humans remains strikingly high.

The general scientific aim of this PhD thesis was to develop an intervention measure that consistently reduces *C. jejuni* numbers in the ceca of broiler chickens by a 1000-fold, which would lead to a reduction in the number of human campylobacteriosis cases by 90%.

Fatty acids are known to possess marked antibacterial activity against a wide range of micro-organisms. Therefore, in the first experimental study (chapter 3.1) the potential contribution of medium-chain fatty acids (MCFA) to control *C. jejuni* in poultry was evaluated.
In vitro experiments revealed relatively low minimal inhibitory concentrations (MIC) at pH 6 (of 0.25 to 0.5 m*M*) for caproic, caprylic and capric acid against *C. jejuni* compared to other bacterial pathogens (including *Salmonella enterica* serovar Typhimurium). Also, these MIC values were about 10-fold lower compared to those for short-chain fatty acids against *C. jejuni*. Time-kill curve experiments showed marked bactericidal properties for capric acid, especially. Therapeutic administration of free caproic, caprylic and capric acid or micro-encapsulated caproic and caprylic acid in the feed at 1% (wt/wt) for the last three days of a 28-day *in vivo* experiment did not reduce bacterial counts of broilers experimentally inoculated with *C. jejuni* after two weeks. The same was observed for a water application of MCFA, where an emulsified commercial mixture of caproic, caprylic, capric and lauric acid was added to the drinking water at 0.4% (vol/vol). To examine the protective effect of this emulsion, it was added to the drinking water at the same dose from day-of-hatch onward. Five

days after *C. jejuni* challenge of only a few two-week-old birds (seeders), which act as a transmitting source for the remainder of the animals (contact animals), cecal colonization of both seeders and contact animals, and thus transmission, was not reduced. The inability of MCFA to kill *C. jejuni* in the chicken cecum was validated using a cecal loop experiment, where capric acid was directly injected into the ceca of the animals at concentrations of multiple times the MIC, indicating that *C. jejuni* is protected against the antibacterial activity of MCFA in the broiler cecum. *In vitro* tests in the presence of chicken intestinal mucus indicated that the mucus layer plays a crucial role in this protective effect. Therefore, it was concluded that MCFA are not able to exert their anti-*C. jejuni* properties in the chicken gut at relevant doses and thus will not contribute to *C. jejuni* control once the bacterium reached the ceca. In a final *in vivo* trial of the first study, we investigated the effect of MCFA on the *C. jejuni* colonization threshold in chicks. For this purpose, birds were housed individually to eliminate *C. jejuni* transmission between animals of the same group. We demonstrated that adding the abovementioned emulsion of MCFA to the drinking water of individually housed broilers at 0.4% (vol/vol) resulted in less chicks being colonized 24 hours after inoculation with low (3×10^2 colony-forming units (cfu)) to moderate (2×10^3 cfu) *C. jejuni* numbers. In addition, no viable *C. jejuni* bacteria could be recovered from the treated drinking water.
In conclusion, intestinal mucus is at least in part responsible for forming a protective cecal niche, rendering *C. jejuni* unresponsive to the antibacterial effects of MCFA observed *in vitro*. MCFA may, however, be capable to reduce the probability of *C. jejuni* entrance into a flock if exposure is limited, probably by exerting an antibacterial effect in more proximal sites of the gastro-intestinal (GI) tract.

Several plant-derived compounds are known to possess marked antibacterial activity against a wide range of micro-organisms, including *C. jejuni*. Therefore, in a second study (chapter 3.2) a selection of plant-derived antimicrobial compounds (caffeic, gallic, protocatechuic and vanillic acid, epigallocatechin gallate, *trans*-cinnamaldehyde and thymol) were screened for their inhibitory and bactericidal properties against *C. jejuni*. The cinnamon-oil ingredient *trans*-cinnamaldehyde (CIN) and the green tea polyphenol epigallocatechin gallate (EGCG) had the lowest MIC (0.125 m*M*) of the compounds tested. In addition, CIN was already bactericidal at 0.125 m*M* while EGCG was not. For this reason, CIN was tested in a broiler chick seeder model in which it was added to the feed in coated form, to enhance survival along the GI tract, at an effective concentration of 0.3% from day-of-hatch for the entire 22-day duration of the experiment. Eight days after inoculation of the seeder animals with *C.*

jejuni, cecal bacterial counts were not reduced. This observation was confirmed in a cecal loop experiment, where CIN concentrations, much higher than the MIC, did not reduce cecal *C. jejuni* numbers in treated ceca at two and 24 hours after injection. In conclusion, this study shows a marked discrepancy between *in vitro* and *in vivo* activity of *trans*-cinnamaldehyde against *C. jejuni*.

Given the rather disappointing results obtained when using fatty acids or plant-derived antimicrobial compounds, in a third study (chapter 3.3), a completely different approach was chosen: immune-based intervention measures. Studying passive immunization was chosen over direct vaccination, for two reasons: (1) the interaction of *C. jejuni* in chicks is of commensal nature and an inefficient immune response is induced during colonization; (2) maternally-derived antibodies are believed to play a key role in protecting broilers against *C. jejuni* colonization during the first two weeks. This indicates that passive administration of such antibodies may be a promising approach in extending the *C. jejuni*-free status of the flock up to slaughter.

For this purpose, *Campylobacter*-free laying hens were repeatedly immunized with either a whole cell protein lysate or only the hydrophobic protein fraction of *C. jejuni*, using Freund's Complete (first immunization) and Incomplete (booster immunizations) Adjuvant, after which their eggs were collected. Preventive administration of the hyperimmune yolks to the feed of broiler chicks from day seven onward dramatically reduced cecal colonization of seeders, experimentally inoculated with the homologous *C. jejuni* strain at 11 days of age. In addition, transmission to contact animals was significantly reduced (hydrophobic protein fraction) or even completely prevented (whole cell protein lysate). *In vitro* tests using purified immunoglobulin Y (IgY) indicated that *C. jejuni* motility was unaffected but adhesion to chicken intestinal mucus was enhanced, suggesting that IgY promotes mucosal clearance *in vivo*. Western Blot analysis and mass spectrometric analysis identified several of the immunodominant antigens of *C. jejuni*, which may be promising targets for the development of subunit vaccines for *C. jejuni* control in poultry.

To conclude, where the use of fatty acids or plant-derived antimicrobial products will probably not lead to the ultimate goal of reducing the cecal *C. jejuni* load of broiler chicks by a 1000-fold, passive immunization seems a very promising alternative intervention measure to reduce *C. jejuni* colonization in broiler chicks.

SAMENVATTING

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Campylobacter jejuni is vandaag de belangrijkste oorzaak van gastro-intestinale ziekte veroorzaakt door voedselpathogenen bij de mens wereldwijd en vormt een ernstig gevaar voor de volksgezondheid. Kippen worden aanzien als de belangrijkste bron voor campylobacteriose in de mens. *C. jejuni* is aangepast aan de omgeving van de kippendarm en gekolonizeerde vogels kunnen zeer hoge bacterie aantallen herbergen, voornamelijk in de ceca, zonder echter klinische symptomen te vertonen. Vanaf een leeftijd van twee tot drie weken zijn kippen zeer gevoelig voor *C. jejuni* kolonizatie en van zodra de bacterie in een kippentoom wordt geïntroduceerd, leidt dit tot de kolonizatie van alle dieren in de toom op slachtleeftijd. Dit resulteert in een enorme hoeveelheid gecontamineerde karkassen na behandeling in het slachthuis. *C. jejuni*-besmette karkassen kunnen de pathogeen overdragen naar de mens. Aangezien het cecum het enige punt is waar *C. jejuni* vermeerdering mogelijk is doorheen de volledige voedselketen, zouden maatregelen tijdens de primaire productie gericht op het reduceren van de cecale kolonizatie een grote impact kunnen hebben op het aantal campylobacteriosis gevallen bij de mens. Vandaag is er echter geen doeltreffende interventie maatregel voorhanden die *C. jejuni* kolonizatie in de kip consistent kan reduceren. Als gevolg, blijft het aantal humane campylobacteriose gevallen dramatisch hoog.

Het wetenschappelijk doel van dit onderzoek was om een interventie maatregel te ontwikkellen die consistent tot een 1000-voudige reductie leidt van levende *C. jejuni* kiemen ter hoogte van de ceca van braadkippen. Dit zou het aantal campylobacteriose gevallen bij de mens tot tien maal kunnen reduceren (Rosenquist *et al*., 2003, Messens *et al*., 2007).

Van vetzuren is het geweten dat zij een sterke antibacteriële werking uitoefenen tegen een brede waaier aan micro-organismen. Om die reden werd hun mogelijke bijdrage tot de bestrijding van *C. jejuni* kolonizatie bij pluimvee nagegaan.
Hiertoe werd in een eerste studie (hoofdstuk 3.1) de anti-*C. jejuni* eigenschappen van middellange keten vetzuren (MKVZ) nagegaan *in vitro* en werd via verschillende *in vivo* modellen de mogelijkheid van deze zuren om *Campylobacter* kolonizatie in de vleeskip te reduceren onderzocht. Toedienen van deze zuren in hun vrije vorm of ingebed in een matrix, die vrijstelling toelaat ter hoogte van de darm, aan reeds gekolonizeerde kippen leidde niet tot een reductie in cecale *C. jejuni* aantallen. Om het preventieve effect na te gaan werden MKVZ toegediend aan ééndagskuikens tot aan het einde van het experiment. Na twee weken werden

enkele kuikens (seeders) oraal geïnoculeerd met *C. jejuni*. Deze dieren fungeren als transmissiebron voor de rest van de dieren. Vijf dagen na inoculatie, waren de cecale *C. jejuni* aantallen en transmissie naar contact dieren niet gereduceerd ten opzichte van de controle groep.
Beide waarnemingen werden gevalideerd in een cecaal loop model, waar caprinezuur geïnjecteerd werd in de ceca van de dieren aan concentraties ver boven de minimaal inhibitorische concentratie. Dit doet vermoeden dat *C. jejuni* beschermd is tegen de antibacteriële werking van MKVZ in het cecum van de vleeskip. Via *in vitro* testen werd aangetoond dat intestinale mucus van de kip een belangrijke rol speelt in dit beschermend effect. Er werd besloten dat MKVZ niet in staat zijn om hun anti-*C. jejuni* activiteit uit te voeren in het kippen cecum aan relevante concentraies. Als gevolg zal hun bijdrage tot het bestrijden van *C. jejuni* kolonizatie bij pluimvee beperkt zijn eens de bacteriën de ceca hebben bereikt.

Om na te gaan of MKVZ een anti-*C. jejuni* effect veroorzaken in meer proximale delen van het gastro-intestinaal (GI) stelsel (i.e. in de krop en maag), werden vleeskippen individueel gehuisvest om op die manier transmissie van gekolonizeerde naar niet-gekolonizeerde dieren uit te sluiten. Daags na experimentele inoculatie werden de ceca gecontroleerd op aanwezigheid van *C. jejuni*. Preventieve administratie van MKVZ resulteerde in een hogere kolonizatie drempelwaarde. Deze observatie kon echter niet worden gevalideerd in een seeder model, wat de situatie in de kwekerij beter benadert. Er kan daarom besloten worden dat de potentiële bijdrage van MKVZ tot het reduceren van *C. jejuni* kolonizatie in pluimvee eerder klein is.

Ook van verschillende plantaardige componenten is het geweten dat ze een uitgesproken antibacteriële werking kunnen vertonen. Daarom werd in een tweede luik (hoofdstuk 3.2) een selectie van plantaardige antibacteriële componenten gescreened op groei-inhiberende en bactericide eigenschappen tegenover de gebruikte *C. jejuni* stam. Uit de resultaten van de *in vitro* testen bleek *trans*-cinnamaldehyde de meest veelbelovende van de geteste component te zijn. Echter, verschillende *in vivo* experimenten, gebruik makend van een seeder, een cecaal loop en een therapeuthisch model faalden om enige reductie in cecale *C. jejuni* aantallen met zich mee te brengen, zelfs wanneer *trans*-cinnamaldehyde werd ingekapseld om vroegtijdige afbraak doorheen het GI stelsel te minimaliseren.

Gezien de niet echt bemoedigende resultaten die werden bekomen met vetzuren en plantaardige antibacteriële componenten werd in een derde en laatste studie (hoofdstuk 3.3) voor een andere aanpak geopteerd: controle maatregelen gebaseerd op immuniteit. Er werd beslist om in plaats van directe vaccinatie het effect van passieve immunizatie op bestrijding van *C. jejuni* bij pluimvee na te gaan, en dit om twee redenen: (1) de interactie van *C. jejuni* en de kip is commensaal van aard en daarom wordt slechts een inefficiënte immuun respons opgewekt tijdens kolonizatie; en (2) maternale antilichamen worden verondersteld een zeer belangrijke rol te spelen in de bescherming van vleeskippen tegen *C. jejuni* kolonizatie tijdens de eerste twee weken na uitkippen. Dit doet vermoeden dat passieve toediening van dergelijke antilichamen een veelbelovende aanpak kan zijn om de *C. jejuni*-vrije status van braadkippen te verlengen tot slachtleeftijd.

Hiertoe werden *Campylobacter*-vrije legkippen herhaaldelijk geïmmunizeerd met een volledig cellysaat of enkel de hydrofobe eiwitfractie van *C. jejuni* waarna hun eieren werden verzameld. Preventieve toediening van de immunoglobuline Y (IgY)-rijke dooiers aan het voeder van vleeskippen leidde tot een massale reductie in cecale *C. jejuni* aantallen in seeders en leidde tot een drastische reductie (hydrofobe eiwitfractie) of zelfs volledige eliminatie (volledig cellysaat) van transmissie naar contact dieren na experimentele inoculatie met de homologe *C. jejuni* stam. *In vitro* testen met opgezuiverd IgY toonden aan dat *C. jejuni* motiliteit niet werd aangetast maar dat adhesie aan intestinale mucus van de kip gepromoot werd, wat een verhoogde mucosale clearance doet vermoeden *in vivo*. Via Western Blot analyse en massa spectrometrie werden verschillende van de immunodominante antigenen van *C. jejuni* in de kip geïdentificeerd, die mogelijks kunnen worden gebruikt voor de ontwikkeling van subunit vaccins ter bestrijding van *C. jejuni* kolonizatie in pluimvee.

Dus, daar waar het gebruik van vetzuren en plantaardige antimicrobiële componenten hoogst waarschijnlijk niet zal leiden tot een 1000-voudige reductie in het aantal cecale *C. jejuni* bacteriën in vleeskippen, lijkt het gebruik van passieve immunizatie een veelbelovende alternatieve maatregel in het bestrijden van *C. jejuni* kolonizatie bij pluimvee.

CURRICULUM VITAE

Personalia

David Hermans werd op 9 februari 1982 geboren te Gent. Na zijn middelbare studies Wiskunde-Wetenschappen startte hij in 2001 met de Master opleiding Biochemie aan de Katholieke Hogeschool Sint-Lieven te Gent en volgde hij een Master na Master in Medical Molecular Biotechnology aan de Universiteit van Gent, die hij succesvol afrondde in 2008 met grote onderscheiding.

Vanaf oktober 2008 trad hij in dienst als doctoraatsbursaal aan de vakgroep Pathologie, Bacteriologie en Pluimveeziekten, waar hij gedurende vier jaar onderzoek verrichtte naar *Campylobacter* controle bij pluimvee.

David Hermans is auteur van meerdere publicaties in nationale en internationale tijdschriften en hij presenteerde zijn onderzoeksresultaten op een internationaal congres aan de hand van een poster en mondelinge presentatie.

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DANKWOORD

DANKWOORD

Om te beginnen had ik graag mijn promotoren willen bedanken om mij de kans te geven mijn doctoraatsonderzoek uit te voeren aan de vakgroep Bacteriologie. Professor Haesebrouck, bedankt voor het beschikbaar stellen van de labo's en materiaal en voor het nalezen van mijn teksten en doctoraatsthesis. Dankzij uw kritische kijk werden deze naar een hoger niveau getild. Frank, Professor Pasmans, bedankt om in mij te geloven en mij de kans te geven dit doctoraatsonderzoek aan te vatten, en voor de vlotte en snelle verbetering van mijn teksten. Filip (Professor Van Immerseel), bedankt voor het kritisch nalezen van mijn manuscripten en doctoraatsthesis.

Graag had ik ook de leden van de begeleidings- en examencommissie, Professor Dr. L. De Zutter, Dr. G. Rasschaert, Professor Dr. H. De Greve, Professor Dr. A. Martel, Dr. D. Vandekerchove en Dr. J. Zoons willen bedanken voor de interessante en nuttige tips, suggesties en opmerkingen. Mijn dank gaat ook uit naar de dienst contractueel onderzoek van de federale overheidsdienst volksgezondheid, veiligheid van de voedselketen en leefmilieu, met in het bijzonder Dr. Dominique Vandekerchove, voor de financiering van dit project.

Ook een woordje van dank aan de collega's van het instituut voor landbouw- en visserij onderzoek. Marc (Professor Heyndrickx), Winy, Geertrui en Joris: bedankt voor de leuke en vlotte samenwerking! Ook Professor D. Deforce van de faculteit Farmaceutische Wetenschappen had ik graag willen bedanken voor het toegankelijk stellen van zijn labo. Katleen & collega's, heel erg bedankt voor de hulp! Twas altijd leuk om bij jullie te komen werken!

Tijdens dit project werden heel wat *in vivo* proeven uitgevoerd. Bij deze had ik alle mensen die me daarbij hebben geholpen uitvoerig willen bedanken. Uit schrik iemand te vergeten zal ik niet iedereen opnoemen, maar de "vaste" helpende handen Hanne, Nathalie en Marc V verdienen toch een speciale vernoeming. Maar uiteraard ook Ruth, Sofie G en Anke voor de hulp bij het immuniseren van mijn kippen en An M voor de hulp bij de loop experimenten. Sjaoji, thanks for taking care of my chicks during my absence! En last, maar zeker niet least: Kim, bedankt om mij op mindere momenten gemotiveerd te houden en om mij bij te staan tijdens onze 32-uur durende biggetjesproef!

Aan mijn bureaugenoten An G, Gunther en Sjaoji: bedankt voor de leuke momenten! We waren misschien niet de meest luidruchtige bureau, maar daardoor zeker en vast wel de productiefste! ;-)

Miet, jij was de eerste om me 4 jaar geleden welkom te heten. Merci om mij direct thuis te doen voelen! ☺ Ellen en Myrthe, jullie waren leuke buurvrouwen! Ellen, hopelijk kan ik vlug terug beginnen lopen zodat we eindelijk voor die triathlon kunnen gaan! ;-)

Aan Alexander, Bram, Bregje en Elin, Sofie DB, Venessa en Annemieke: bedankt voor de leuke uitjes, moeten we zeker en vast nog eens over doen! Bregje: je was een leuke roommate! Jammer genoeg kon jij niet altijd hetzelfde over mij zeggen ☺ Venessa, bedankt voor de persoonlijke babbel en om je zo braaf te houden in Porto ;-) En aangezien jij waarschijnlijk de volgende bent om te doctoreren: veel succes! Je zal da zeker goed doen!

Aan de mannen van het secretariaat, Gunter, Jo en Koen: bedankt om ons steeds bij te staan met allerhande problemen, bestellingen en andere administratieve rompslomp. Merci Gunter voor het regelen van de skireizen. Alex, we moeten zeker nog eens terug om die zwarte piste te gaan temmen. Elin, ik stel voor dat we ons onvergetelijk moment bij die ene keer houden ☺

Ook aan alle andere collega's: Katleen, Melanie, Lien VDM, Nele, Maxime, Yang, Jungang, Guangzhi, Cheng, Silvana, Lien DC, Ilse, Lieven, Tom, Connie, Pascale, Mark, Serge, Arlette, Marleen, Filip B, Caroline, Iris, Roel, Mojdeh, Erna, Magda, ...: merci allemaal voor de leuke tijd!

Ook de collega's van Pathologie en Morfologie wil ik zeker niet vergeten. Ruth, Celine, Evi, Wolf, Marjan, Dorien, Sofie G, Sofie K, Karen, Stefanie, Leen en Annelies en Marlien: bedankt voor de leuke babbels! Uiteraard een apart woordje voor Ruth, mijn vaste kippenvriendin ☺. Merci voor de hulp en voor de babbels (slash roddels ☺), kzal ze echt missen ☹. Karen, merci om mijn virus weg te krijgen en mijn doctoraat te redden! ;-) En Wolf, respect man om het vol te houden tussen al dat vrouwelijk geweld ;-)

Tenslotte wil ik nog wat ex-collega's bedanken. Anja R, jammer dat je zo vlug bent vertrokken ☹ Misschien tot op graspop? Hanne en Anja VDB, merci voor de hulp in het labo en voor de leuke quiz-avonden! Ge weet het hé, als jullie in Brugge zijn... ;-) Anja, merci voor alles, maar vooral voor uw balonnen! ☺ Ze leven nog altijd trouwens...

Aan mijn thesisstudente Jolien: merci voor de vrolijke noot tijdens mindere tijden! ;-)

Aan mijn familie en vrienden: bedankt voor jullie steun tijdens deze periode, en vooral tijdens de laatste maanden. Loortje, merci om het met mij uit te houden! Kweet dat ik soms niet uit te staan was de laatste tijd maar dat is nu helemaal voorbij! Nuja, helemaal... ☺